An investigation of the prebiotic potential and gut health benefits of Irish Seaweeds

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Doctor of Philosophy

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at University College Cork or elsewhere

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Abbreviations.

ACE-1	Angiotensin-converting enzyme 1
ACC	Acetyl-CoA carboxylase
AcFu	Acetylated fucoidan
Ae	*Alaria esculenta*
AHR	Airway hyper-responsivness
ALD	Atrial aldosterone
ALT	Alanine transaminase
AMP	Adenosine monophosphate
An	*Ascophyllum nodosum*
ARE	Antioxidant-response element
BAL	Bronchoalveolar lavage
Bcl-2	B cell lymphoma – 2
BHA	Butylated hydroxyanisol
BHI	Brain Heart Infusion
BHT	Butylated hydroxytoluene
Cc	*Chondrus crispus*
C/EBPα	CCAAR/enhanced-binding protein α
CDC	Centre for Disease Control
Cf	*Codium fragile*
CFA	Conjugated Fatty acids
CFU	Colony forming unit
COX-2	Cyclooxygenase-2
CPV	Circulating plasma volume
CVD	Cardiovascular disease
DF	Dietary Fiber
DHA	Docosahexaenoic acid
DOCA	Deoxycorticosterone acetate
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
DR	Diabetic retinopathy
Fse	*Fucus serratus*
Fsp	*Fucus spiralis*
Fv	*Fucus vesiculosus*
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinases
F6PK	Fructose-6-phosphate phosphoketolase
FADD	Fas-Associated protein with Death Domain
FAK	Focal Adhesion Kinase
FFAR	Free Fatty Acid Receptor
FISH	Fluorescent in-situ hybridization
FOS	Fructooligosaccharide
G1	Growth 1
GAE	Gallic acid equivalents
G+C content	Guanine + cytosine

GE	Glucose equivalents
GIT	Gastrointestinal tract
GJIC	Gap Junctional Intercellular Communication
GLP	Glucagon-like peptide
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOS	Galactooligosaccharide
GRAS	Generally Regarded As Safe
GPR	G-protein-coupled Receptor
HCII	Heparin cofactor II
He	*Himanthalia elongata*
HPLC	High-performance Liquid Chromatography
IC50	Half maximal inhibitory concentration
IgE	Immunoglobulin E
IGF-1	Inulin-like growth factor 1
i.p.	Intraperitoneal
IRS-1	Insulin receptor substrate 1
ITF	Inulin-type fructans
KEAP1	Kelch-like ECH-associated protein 1
LBS	*Lactobacillus* select media
LCC	Lewis lung carcinoma cells
Ld	Laminaria digitata
Lh	*Laminaria hyperborea*
LjGP	*Laminaria japonica* glycoprotein
LMW	Low molecular weight
LWMP	Low molecular weight polysaccharides
L-PA	Low molecular weight alginate
MMP	Matrix metalloproteinase
MRS	de Man, Rogosa, Sharpe
NAOS	Neoagaro-oligosaccharides
NCD	Non-communicable disease
NDO	Non-Digestible Oligosaccharide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
OD	Optical density
OVA	Ovalbumin
Nrf2	Nuclear factor E2-related factor 2
PARP	Poly (ADP-ribose) polymerase
Pc	*Pelvetia canaliculata*
PCA	Principal Coordinate Analysis
PEP-PTS	Phospohenolpyruvate-phosphotransferase
PI3K	Phosphoinositide 3-kinase
Pk	Phosphoketolase
Pp	*Palmaria palmata*

PPARγ	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated Fatty Acids
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
RTE	Ready-to-eat
SCFA	Short chain fatty acids
SE	Standard error
Sl	*Saccharina latissima*
SQDG	Sulfoquinvosyldiacylglycerol
TB	Tuberculosis
TBHQ	Tert-butylhydroquinone
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TPC	Total Phenolic Content
TPP	Thiamine diphosphate-dependent
TNF-α	Tumour necrosis factor – α
Ui	*Ulva intestinalis*
UV	Ultraviolet
UVB	Ultraviolet – B radiation
VEGF	Vascular endothelial growth factor
W/W	Weight per weight
WHO	World Health Organisation
XOS	Xylooligosaccharide

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Thesis Abstract.

Seaweeds are a large and diverse group of marine organisms that are commonly found in maritime regions. The term 'seaweed' itself does not have any taxonomic value, but is rather a popular term used to describe large attached (benthic) marine plants found in the groups that are referred to as the brown seaweeds (phylum Ochrophyta, class Phaeophyceae), the red seaweed (phylum Rhodophyta) and the green seaweeds (phylum Chlorophyta). To date, about 6000 species of seaweeds have been identified. Seaweeds are an excellent source of biologically active secondary metabolites and have been shown to exhibit a wide range of therapeutic properties, including anti-cancer, anti-oxidant, anti-inflammatory and anti-diabetic activities, while also being a potential source of prebiotics. As such, seaweeds have been used to treat a wide variety of health conditions such as cancer, digestive problems, and renal disorders. Today, increasing numbers of people are adopting a "westernised lifestyle" characterised by low levels of physical exercise and excessive calorific and saturated fat intake. This has led to a rise in chronic non-communicable diseases (NCDs) such as cancer, cardiovascular disease, and *diabetes mellitus*, being reported. Recently, NCDs have replaced communicable infectious diseases as the number one cause of human mortality. Current medical treatments for NCDs rely mainly on drugs that have been obtained from the terrestrial regions of the world, with the oceans and seas remaining largely an untapped reservoir for exploration. The focus of Chapter 1 is the potential of using seaweed derived bioactives including polysaccharides, antioxidants and fatty acids, amongst others, to treat chronic NCDs such as cancer, cardiovascular disease and *diabetes mellitus*. The literature review presented here clearly demonstrates the plethora of novel bioactives that seaweed has to offer. While much of the research heralds the therapeutic effects from *in vitro* studies, the way is being laid to assess their efficacy *in vivo* through extensive animal trials and human clinical studies.

Seaweeds are well regarded as source of dietary fibres and polysaccharides, that contain complex glycosidic linkages that the human hydrolytic degrading enzymes cannot break down. Seaweeds are a putative novel source of prebiotic compounds. A prebiotic is a selectively fermented food ingredient that promotes specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health. The most well-known beneficial genus is *Bifidobacterium*.

In Chapter 2, the bifidogenic potential of cold-water extracts from 15 Irish seaweed species was investigated using an anaerobic fermentation/*ex vivo* approach. A *Bifidobacterium* minimal growth media was prepared, containing all the required nutrient for bifidobacteria to grow except for the presence of a carbohydrate source. Five *Bifidobacterium* species (*B. breve, B. lactis, B. longum, B. bifidum, B. infantis)* were tested for their ability to use seaweed carbohydrates as their sole carbon source for growth. At a concentration of 2.5 mg/ml, 11 out of 15 seaweeds were observed to significantly ($p<0.05$) stimulate the growth of at least one of the *Bifidobacterium* species at both experiment time points (20 h and 28h). The greatest effect was found with the brown seaweeds, *Fucus serratus* and *Pelvetia canaliculata*, with *F. serratus* being chosen for further evaluation. Significant bifidogenic activity was detected with *F. serratus* at both 1.25 mg/ml and 0.625 mg/ml for *B. breve*, *B. lactis* and *B. longum.* It was also shown that, at 1.25 mg/ml, *F. serratus* had a significantly greater stimulatory effect on the growth of *B. longum* than the FOS prebiotic control. Interestingly, appreciable levels of phenolic compounds were observed in several of the seaweed extractions including *F. serratus*. High levels of such compounds are known to have antimicrobial activity and may explain an inhibitory effect observed with the *F. serratus* at 2.5 mg/ml. The results of this study indicate that seaweed extracts are good candidates for further prebiotic investigation, however a refined extraction process and experimental methodology should be utilised.

In Chapter 3, a polysaccharide-rich extract was prepared from *F. serratus* (the Fse extract) and examined for prebiotic potential using an anaerobic *ex vivo* faecal fermentation. Samples were taken at 0 h, 5 h, 10 h, 24 h, 36 h and 48 h for DNA sequencing, enumeration of *Bifidobacterium* and *Lactobacillus*, short-chain fatty acid (SCFA) analysis and hydrogen sulphide production. SCFA production is a major indicator of prebiotic stimulation in the colon. Here, a 1.5-fold increase was observed in the production of SCFAs, particularly in the production of propionate (2.3-fold increase) and acetate (1.4-fold increase). There was also an associated significant increase ($p<0.05$) in the ratio of propionate production, rising from 15% in the control to 24%. There was no significant increase butyrate concentration. DNA sequencing analysis revealed that the Fse extract had no notable effect on the abundance of *Bifidobacterium* or *Lactobacillus*. There were, however, notable increases in several

propionate producers such as the genus *Parabacteroides*, the family *Veillonellaceae* and the family *Erysipelotrichaceae*, which is peripherally related to the butyrate-producing superfamily *Lachnospiraceae*. These results indicate that a crude polysaccharide extract from the seaweed *F. serratus* can significantly modulate the activity of the gut microbiota, and alter the SCFA production profile by stimulating propionate and acetate producing members of the microbiota. While the Fse extract did not exhibit all the expected prebiotic markers an increase in the production of SCFA, especially propionate, is a positive outcome in terms of promoting overall gut health and in combating obesity through enhancing satiety. Also, as the extract was shown to withstand gastric digestion, *F. serratus* would be an excellent source of dietary fibre as part of a healthy balanced diet.

The proper function of dietary polysaccharides is greatly dependent on their molecular weight, with low molecular weight oligosaccharides often providing better sources of carbon and energy for bacteria than their parent sugars. The fibre content of seaweeds is typically high molecular weight and while some demonstrate fermentative capacity in the lower intestines most pass through the gut too quickly for the gut microbiota to utilize to any great extent. In Chapter 4, two similarly processed extracts from the brown seaweed *L. digitata* were prepared to study the effect of depolymerisation on prebiotic potential. The fermentation of both extracts brought about a significant increase ($P>0.05$) in total SCFA concentration and the production of the biologically significant SCFAs, butyrate (1.7-fold and 0.9-fold increases), propionate (3.3-fold and 3.1-fold increases) and acetate (1.76-fold and 1.9-fold increases). It was found that the depolymerisation of *L. digitata* with hydrogen peroxide significantly increased the production of propionate and significantly reduced the production of butyrate in comparison with the non-depolymerised extract. Modulation of polysaccharide chain length can bring about different fermentation profiles from the same seaweed raw material and starting faecal microbiota. This could potentially allow the production of ‘designer’ extracts whose impact on the gut microbiota can be predicted beforehand.

Chondrus crispus is an economically and ecologically important red seaweed with a long tradition of usage on the island of Ireland. Red seaweeds, such as *C.*

crispus, contain large quantities of complex polysaccharides such as carrageenans, agarans and xylans which also resist degradation in the gastrointestinal tract and reach the colon intact. As such they can be considered dietary fibre and a possible source of prebiotics. In Chapter 5, a depolymerised polysaccharide-rich extract from *C. crispus* was investigated for prebiotic potential using an anaerobic *ex vivo* faecal fermentation. Significant increases ($p<0.05$) in the production of total short-chain fatty acids, particularly the biologically important SCFAs propionate was recorded. However, there was no significant alteration in the molar ratio of SCFA production or impact on the production of butyrate. High-throughput DNA sequencing revealed that there was no notable impact on the relative abundance of the major probiotic genera of *Bifidobacterium* and *Lactobacillus* and had a minimum effect on the overall microbial population. Increases in propionate concentration, while statistically significant ($p<0.05$) at certain time points, fell short of the levels that would be expected from a putative prebiotic. The conclusion of this study is that polysaccharide extracts from *C. crispus*, as prepared here, are not an appropriate candidate for future prebiotic investigation as only a minimal stimulatory effect on the *ex vivo* microbial population was observed and limited changes in SCFA production.

Food-borne illnesses are a major burden on health services throughout the world. A variety of antimicrobial agents have been used to prolong the shelf-life of processed goods and inhibit the growth of food-borne pathogens but resistance to traditional antimicrobials is spreading quickly. *Listeria monocytogenes* is among the most prevalent food-borne pathogen and is the causative agent of listeriosis. In Chapter 6, the antimicrobial activity of ten ethanol seaweed extracts was examined against a selection of food-borne pathogens including *Listeria monocytogenes*. Extracts from the seaweeds *Fucus vesiculosus, F. serratus F. spiralis, Ascophyllum nodosum* and *Pelvetia canaliculata* significantly inhibited ($p<0.05$) the growth of *Listeria monocytogenes 5788* at 24 h. The *F. vesiculosus* extract was chosen for further evaluation. Molecular weight fractions of *F. vesiculosus* were tested against several listeria stains. It was found that the anti-listerial activity was concentrated in the 0-3.5 kDa and the 3.5-100 kDa molecular weight subfractions, with the 3.5-100 kDa exhibiting the highest activity of all. Antimicrobial activity was found to be positively correlated with high levels of phenolic content. *F. vesiculosus* was found to have the highest level of phenolic contents of all the seaweed species tested at 138.3 ± 0.7 µg

GAE mg^{-1} and the most potent antimicrobial activity. Subfractions from *F. vesiculosus* that exhibited activity against *L. monocytogenes* were also found to contain large quantities of phenolic compounds. The results of this study indicate that the several Irish seaweeds display antimicrobial activity which is correlated to the presence of large concentration of phenolic compounds. *F. vesiculosus* had the highest levels of phenolic compounds of all the seaweeds investigated and demonstrated the most potent antimicrobial activity against the pathogen *L. monocytogenes.* This is a promising outcome as to the potential of utilising such extracts from seaweeds in food products to serve as both biopreservation agent and antioxidants.

Studies, like the one presented here, function to expand our knowledge seaweed as a resource and broaden our understanding of how its potential can be unlocked. The concept of prebiotics is an intriguing one. The delivery of non-digestible components to the gut, after having survived gastric transit, and then being utilised by certain beneficial members of the microbiota, namely *Bifidobacterium* and *Lactobacillus*. However, not all putative prebiotics are made equally and they vary considerably in their fermentability potential. We have shown that seaweed polysaccharides, especially those from brown seaweeds such as laminarin and fucoidan, have great potential. The definitive test of the prebiotic potential of a substrate is to transition from ex vivo to *in vivo* animal trials in small animals and subsequently in large animals/humans. However, several challenges exist in the upscaling of prebiotic studies. As the yield of polysaccharides can be very low following acid hydrolysis, large quantities of seaweed would need to be collected, stored and processed to produce sufficient extracts for further investigation. Additionally, the concentration and type of seaweed polysaccharide can vary greatly depending on the season of collection and the species of seaweed and must be taken into consideration in the production of the product.

Chapter 1

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Looking Beyond the Terrestrial: The Potential of Seaweed Derived Bioactive to Treat Non-Communicable Diseases.

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1.1 Abstract.

Seaweeds are a large and diverse group of marine organisms that are commonly found in maritime regions. They are an excellent source of biologically active secondary metabolites and have been shown to exhibit a wide range of therapeutic properties, including anti-cancer, anti-oxidant, anti-inflammatory and anti-diabetic activities. Several Asian cultures have a strong tradition of using different varieties of seaweed extensively in cooking as well as in herbal medicines preparations. As such, seaweeds have been used to treat a wide variety of health conditions such as cancer, digestive problems, and renal disorders. Today, increasing numbers of people are adopting a "westernized lifestyle" characterised by low levels of physical exercise and excessive calorific and saturated fat intake. This has led to an increase in the numbers of chronic Non-Communicable Diseases (NCDs) such as cancer, cardiovascular disease, and *diabetes mellitus*, being reported. Recently, NCDs have replaced communicable infectious diseases as the number one cause of human mortality. Current medical treatments for NCDs rely mainly on drugs that have been obtained from the terrestrial regions of the world, with the oceans and seas remaining largely an untapped reservoir for exploration. This review focuses on the potential of using seaweed derived bioactives including polysaccharides, antioxidants and fatty acids, amongst others, to treat chronic NCDs such as cancer, cardiovascular disease and *diabetes mellitus*.

1.2 Introduction.

Seaweeds are an extensive group of autotrophic organisms that have a long fossil history. They are globally distributed and can be found in every climatic zone ranging from warm tropical waters to the freezing cold polar regions [1]. At present, more than 10,000 different species of seaweed are known [2]. The traditional division of the various seaweed species is one based largely on differences in pigmentations. The three main groupings are; the brown seaweeds (phylum Ochrophyta, class Phaeophyceae), the red seaweed (phylum Rhodophyta) and the green seaweeds (phylum Chlorophyta) (Figure 1) [3]. Seaweeds have been extensively used by mankind since the beginnings of recorded history in a wide assortment of ways. They are an important source of unique polysaccharides (agar, carrageenan, alginates, *etc.*) for the pharmaceutical and food industries and the use of seaweed extracts as gelling agents and thickeners goes back almost half a millennium. The practice of extracting agar from seaweed was first described in 1658 in China and agar is well known today as a common substrate in bacterial culture media, being first used by the pioneering German microbiologist Robert Koch [1]. Drift seaweed washed up on shore has been used as an organic agricultural fertiliser in coastal regions of the world for centuries. The application of seaweed as a fertiliser improves soil structure, provides trace elements and growth activators [4] as well as resulting in earlier seed germination, the enhancement of crop performance and yield and a better resistance to both biotic and abiotic stresses [5].

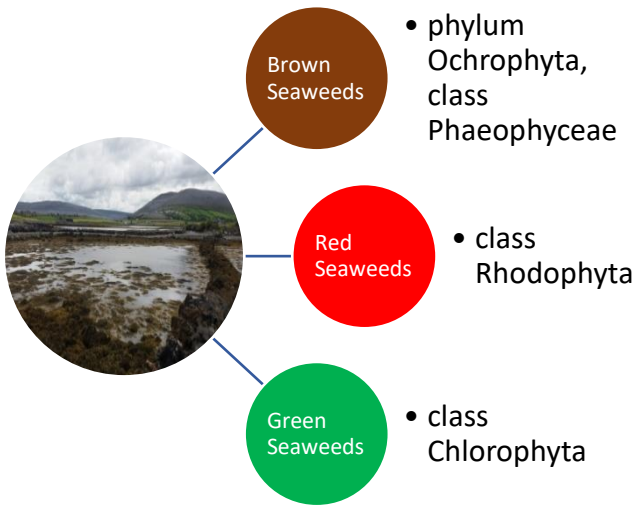


Figure 1.1 Seaweeds are divided into three main groupings based largely on their pigmentation. The groupings are brown seaweeds, the red seaweeds and the green seaweeds.

Since ancient times, edible seaweed species have formed an important part of the culinary tradition in countries of the Far East, such as China, Korea and especially Japan[6]. Seaweed is a very healthy food source with some varieties containing higher levels of minerals and trace elements than terrestrial plants and animal products [7-10]. For examples, a 100 g portion of seaweed can exceed the RDA value for vitamin A, B2 and B12 and two thirds of the vitamin C daily requirement [11]. The protein content of seaweed can vary greatly depending on many factors, such as the season when the plant is harvested and surrounding environmental conditions. Species of red seaweed can contain as much as 21–47 g/protein/100 g dry weight, while brown seaweeds have a comparatively low protein content of 7–16 g/100 g dry weight [12]. Seaweeds are also the best natural source of iodine and their addition to the diet could help people who are lacking in iodine to meet their daily iodine requirements [13]. In traditional Japanese cooking, edible seaweeds are extensively used as a sea vegetable and can also be used as condiments, seasonings and wrappings for sushi [14]. In such ways, they can account for as much as 25% of the daily food intake of some Japanese people [15]. Indeed, every year, over 1.6 kg of dry seaweed is consumed in Japan, on average per person [10]. Some of the more common seaweeds used in food preparation include the brown seaweed species, *Laminaria* (kombu), *Undaria* (wakame) and *Hijiki* (hiziki), and varieties of the red seaweed, *Porphyra* (nori). Edible seaweeds contribute few calories to the diet, owing to their low fat content and because seaweed derived carbohydrates and proteins cannot be fully digested in the gut by human intestinal enzymes [16]. As such, seaweeds are a good source of dietary fibre, which can positively affect satiety in between meals and glucose uptake from food [17]. Furthermore, soluble polysaccharide found in seaweeds may have a prebiotic effect, by stimulating the growth and/or activity of beneficial members of the microbiota such as the *Bifidobacterium* and *Lactobacillus* [18]. While seaweeds have undoubtedly been used extensively for thousands of years in Asia, South America and Oceania as a food source, the culinary use of seaweed has traditionally been very limited in both Europe and North America [1]. Despite this, the use of seaweed as sea vegetables has become more common in Western countries in recent decades [19] as a result of increasing globalization and improved accessibility of Asian cuisine to the rest the world. Furthermore, consumers in developed Western countries are increasingly turning to products from natural sources, including seaweeds [8, 20].

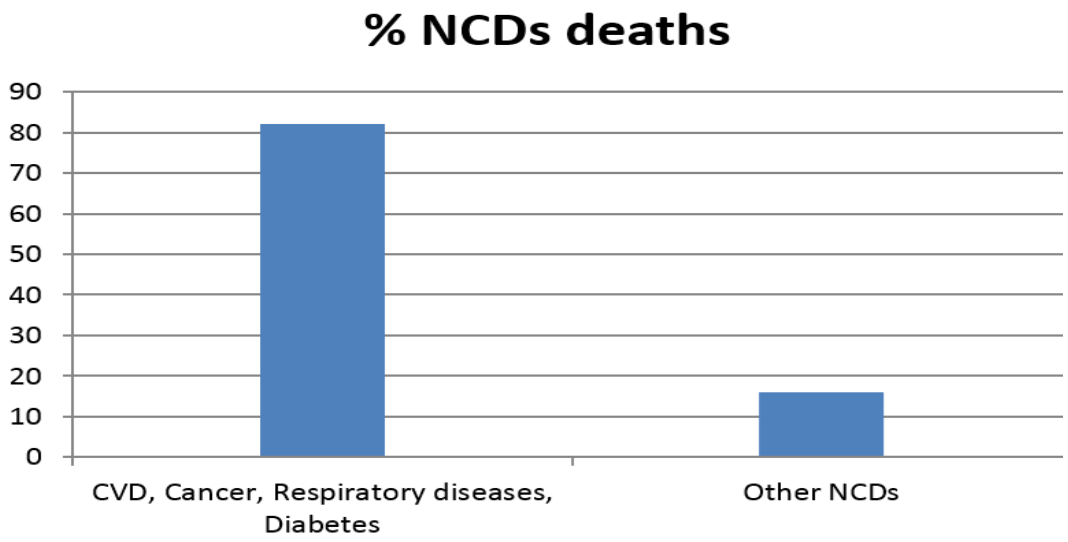
Seaweeds are consistently exposed to both biotic and abiotic pressures in their natural marine environments. These pressures exert an influence on the plant's physiology that leads to the production of metabolites in order for the plant to survive and thrive. Some of these metabolites may act as bioactive components, and thus have potential for use in the development of new functional ingredients and medical treatments. Indeed, secondary metabolites known to be produced by seaweeds have demonstrated therapeutic properties including anti-cancer, anti-oxidant, anti-inflammatory, and anti-diabetic activities [16]. Historically, Asian civilizations have used seaweeds for various medicinal purposes by boiling the seaweed in water and using the decoction as a drug. Japanese and Chinese practitioners have been recorded using seaweeds in herbal medicines as far back as 300 BC. The range of ailments reported to have been treated with seaweed or seaweed derived products is much varied. They include treatments for cancer, digestive problems, dropsy, eczema, glandular problems, goitre, gout, hyper-throidisms, parasitic infection, swollen and painful scrotum and urination and renal disorders [21-23]. In this regard, these metabolites may potentially lead to useful leads in the development of new functional ingredients and medical treatments [16].

The aim of this review is to examine the literature regarding the use of seaweed derived bioactive metabolites in relation to the treatment/prevention of a particular set of diseases referred to as chronic non-communicable diseases (NCDs). NCDs are an extensive group of conditions that, unlike bacterial and viral infections, are not transmissible from person to person. NCDs are a leading cause of death and disability, and affect millions of people globally each year. These long-lasting conditions have a protracted duration and a generally slow rate of progression. The four main types of chronic NCDs are cardiovascular diseases (CVDs), cancer, diabetes mellitus and chronic respiratory ailments [24]. Chronic diseases typically begin to manifest in middle age, following long term exposure to a plethora of unhealthy activities, such as excessive alcohol consumption, primary and secondary smoke inhalation, low levels of physical activity, and the consumption of a diet with excess fat and red meat and low in fibre. The incidence of chronic diseases rises sharply as people start to age, with most people over the age of 65 having a chronic ailment of one sort or another. Today, NCDs are the leading cause of death and disability in the world (Figure 1.2) and are responsible for double the sum total of deaths caused by all infectious deaths

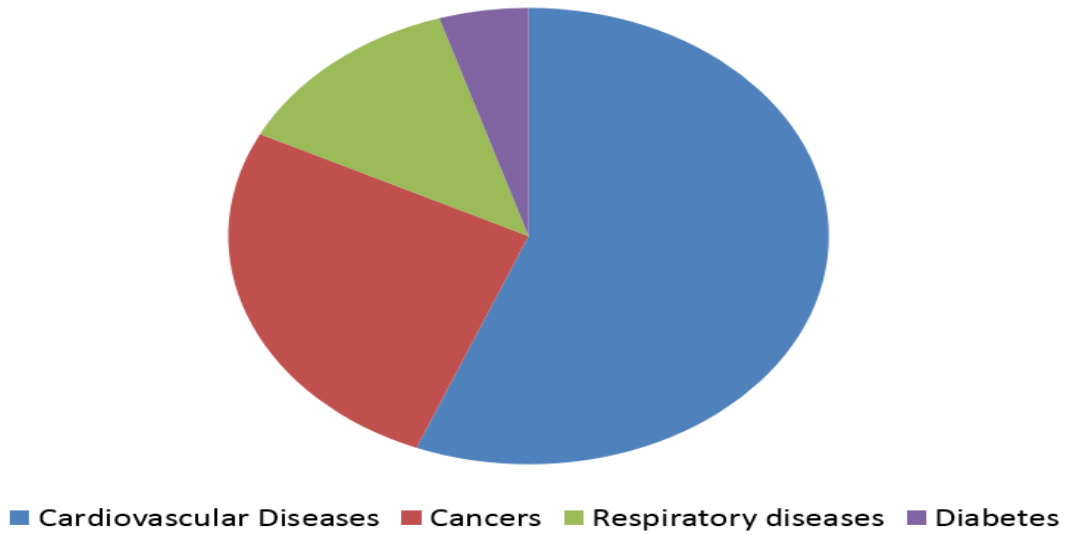
(including HIV/AIDS, TB and malaria), maternal and perinatal conditions and nutritional deficiencies [25]. Consequently, interventions to prevent and control NCDs are essential and since seaweed is an abundant and natural resource with proven therapeutic effects, its contribution to the alleviation of chronic diseases are evaluated henceforth.

Figure 1.2 According to the World Health Organization (WHO) (a) cardiovascular diseases, cancers, respiratory diseases and diabetes account for 82% of all deaths attributed to non-communicable diseases; (**b**) Each year, cardiovascular diseases account for 17.5 million deaths, cancers (8.2 million), respiratory diseases (4 million) and diabetes (1.5 million) [24].

(a)



(b)



1.3 Anticancer Activity of Seaweed Components.

Cancer is a generic name given to a large group of clonal malignant diseases. The defining characteristic of cancer is the rapid creation of abnormal cells that proceed to grow beyond their normal boundaries, leading to the invasion of adjoining areas of the body culminating in the spread of the cancer in a process termed metastasis. Despite a better understanding of cancer biology in the last few decades, the treatment of most cancers has not progressed, with the reduction in cancer deaths mainly being attributed to early detection and preventive measures, rather than new cancer treatments coming on stream [26, 27]. The challenge of developing effective treatments for cancer has encouraged the development of new drugs from natural sources, with seaweeds and the marine environment as a whole expected to be a major frontier in both pharmaceutical and medical cancer research [28].

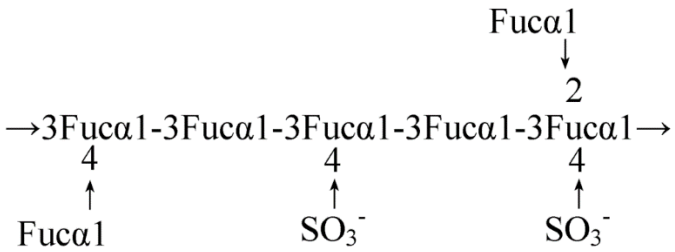
1.3.1 Seaweed-Derived Polysaccharides.

Seaweeds, especially brown seaweeds, are rich in biologically active polysaccharides that exhibit a broad spectrum of biological activities. Examples of these polysaccharides include fucoidans, laminarins and alginic acids [29]. Fucoidans (fucans) are highly sulphated cell-wall polysaccharides found in species of brown seaweeds. Each different brown seaweed species produces its own array of fucans that have unique structural properties, which can be further altered by any number of biotic and abiotic factors to which the seaweed is exposed, as well as the extraction and purification method used to collect the fucan [30]. The biological activity of fucoidans is related to their molecular structure, which include fucose linkage, the sugar type, sulphate content, with molecular weight being the most important determinant. Fucoidan from *Fucus vesiculosus* (Phaeophyceae) is mainly composed of α-(1-3) linked sulphated L-fucose (Figure 3). In *Ascophyllum nodosum* (Phaeophyceae), α-(1-3) linked fucose with low proportion of α-(1-4) linked fucose or a repeating α-(1-3) and α-(1-4), has been reported. Linkages of α-(1-3) found in other polysaccharides have a stronger anticoagulation ability than the α-(1-4) configuration. The sulphate content of fucoidan also influences the anti-cancer and anticoagulant activities. Over sulphated fucoidan has a better α-amylase inhibitory activity than native fucoidan. Furthermore, the location of a sulphate group on fucose could also affect the biological

function of fucoidan. A molecular weight that is too high may result in low solubility and processability, resulting in poor penetration of the cell. Low molecular weight (LMW) fucoidan degraded by gamma-irradiation was shown to increase cell cytotoxicity in comparison to native fucoidan in cancer cell lines such as AGS, MCF-7 and HepG-2. Gamma irradiated fucoidan also showed a higher level of cell transformation inhibition, resulting in higher anti-carcinogenic activity [31, 32]

Evidence suggests that fucoidan can act as an anti-cancer agent through modulation of the human immune system. Fucoidan has been found to induce the maturation of dendritic cells and, in association with other cytokines, to shape the immune responses that are mediated by T-cells. For information of the proposed mechanism of fucoidan bioactivity see Figure 4. Dendritic cells are antigen-presenting cells that play a vital role in effectively stimulating the immune response as they are responsible for the initiation and polarization of adaptive immunity. Data suggest that fucoidan can modulate dendritic cell differentiation and drive it towards a Th1-polarizing phenotype, which could possibly be used in dendritic cell based vaccines for cancer immunotherapy [33]. Polysaccharides isolated from plants and algae have been reported to enhance macrophage activation through specific membrane pattern recognition receptors. These receptors recognize foreign ligands such as those found on carbohydrates during the innate immune response. The major receptors reported for polysaccharide recognition in macrophages are Toll-like receptor 4 (TLR4), CD14, complement receptor type 3 (CR3) and scavenger receptor (SR). Acetyl fucoidan isolated from commercially cultured *Cladosiphon okamuranus* (Phaeophyceae) induced macrophage activation in the murine macrophage cell line, RAW 264.7 through membrane receptors TLR4, CD14 and SRA (anti-scavenger receptor class A) and MAPK signaling pathways [34].

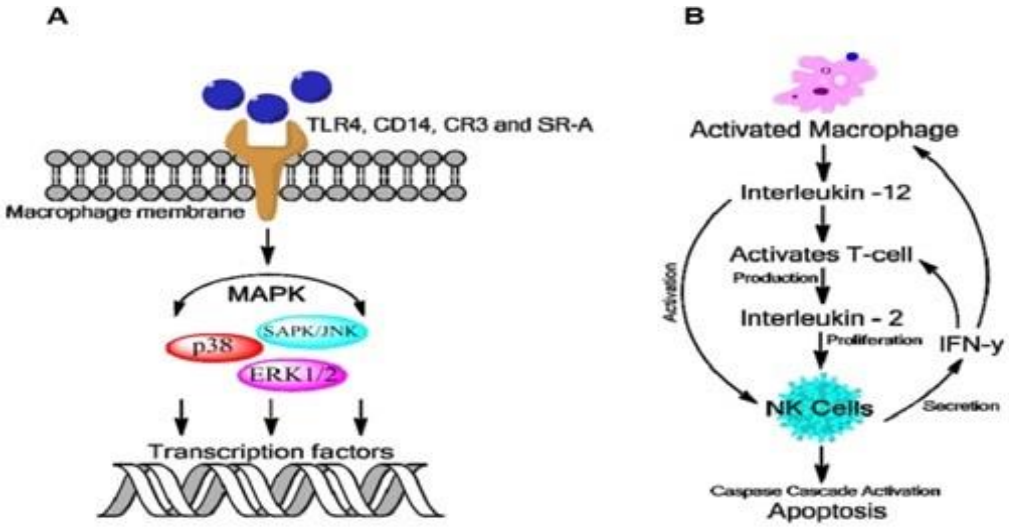
Figure 1.3 Model for the average structure of fucoidan from *Fucus vesiculosus*. The core region of the fucan is composed primarily of a polymer of α (1-3) linked fucose with sulphate groups substituted at the 4 position on some of the fucose residues [35].



Fucoidan has also been shown to have cyto-protective properties. Chemotherapeutic anticancer drugs are effective against cancer cells but, because of a lack of selectivity, they also attack normal immune cells. It has been demonstrated that fucoidan can protect dendritic cells from the effect of 5-Fluorouracil (a representative cancer drug) [36]. Studies performed *in vitro* with crude fucoidan extracted from *Sargassum* sp. and *F. vesiculosus* demonstrated a reduction of the viable number of Lewis lung carcinoma cells and melanoma B16 cells in a dose dependent manner. Exposure to the fucoidan also caused morphological changes in the melanoma cells, which were indicative of apoptosis being induced. When male mice were challenged with daily i.p. injections of crude fucoidan from either seaweed over a 4 day period, the cytotoxic activity of their natural killer cells was enhanced [37]. Fucoidan extracted from the sporophyll of *Undaria pinnatifida* (Phaeophyceae) was reported to show anti-tumour activity against PC-3, HeLa, A549 and HepG2 cell lines, which was comparable to that of commercially obtained fucoidan [38]. Fucoidan from *Saccharina cichorioides* (Phaeophyceae), *Fucus evanescens* (Phaeophyceae), and *U. pinnatifida* was investigated for effects on proliferation, neoplastic formation, and colony formation of mouse epidemial cells (JB6 C141), human colon cancer cells (DLD-1), breast cancer cells (T-47D) and melanoma (RPMI-7951). These particular fucoidans specifically and significantly suppressed the proliferation of human cancer cells and exhibited less cytotoxicity towards normal mouse epidermal cells [39]. Another investigated the possibility of using acetylated fucoidan (AcFu) nanoparticles loaded with the chemotherapy drug doxorubicin for the treatment of cancer using the cell lines HCT-116 and HCT-8. The nanoparticles demonstrated first-order drug release for 5 days following treatment. Treated macrophages were found to overexpress various anti-tumour cytokines, such as TNF-α and GM-CSF. The AcFu

particles were also resistant to the multidrug resistant characteristics of cancer cells [40].

Figure 1.4 Proposed mechanism for fucoidan bioactivity (A) Macrophage activation by fucoidans as mediated through specific membrane receptor activation namely TLR-4, CD14, CR-3 and SR which induces intracellular signaling via mitogen-activation protein kinases (MAPKs); (B) Activation of macrophages leads to the production of cytokines such as IL-12, IL-2 and IFN-γ which enhance NK cell activation that may stimulate T-cell activation[41].



The laminarins are a group of water soluble polysaccharides produced by brown seaweeds. They consist of 1,3- and 1,6-linked β-D-glucose residues and normally have a molecular weight of 4–5 kDa. Laminarin isolated from *Eisenia bicyclis* (Phaeophyceae) was shown to inhibit human melanoma SK-MEL-28 and colon cancer DLD-1 cells. It was also demonstrated that decreasing the molecular weight of the laminarin (DP: 9–23) and increasing the ratio of 1–6 linked glucose residues increased the anticancer activity [29]. Rats fed a diet of 2% (*w*/*w*) laminarin suppressed indole, p-cresole and sulphide production significantly. Such compounds are produced from proteins by colonic bacteria and are putative risk markers for the development of colon cancer [42]. Other studies have also reported anti-cancer activity of laminarins and fucoidans [43]. One investigated the effectiveness of using polysaccharides from the edible *Sargassum latifolium* (Phaeophyceae) in chemoprevention. Fractions of water soluble polysaccharides from *S. latifolium* were tested for their chemopreventive efficacy revealing a range of chemopreventive properties, including anti-initiating, anti-promoting, and inhibition of NO, TNF-α and COX-2 [44]. A hot water-soluble polysaccharide from *Capsosiphon fulvescens* (Chlorophyta) showed significant

inhibition of human cancer cells in a dose dependent manner. Treated cells exhibited a marked increase in caspase-3 activation, and decreases in both the expression of Bcl-2 and the phosphorylation of insulin-like growth factor-I (IFG-1) receptor. Treatment with the polysaccharide extract also decreased the recruitment of p85 to IGF-1 receptor and insulin receptor substrate-1 (IRS-1)[21]. Sulphated polysaccharides from the thallus of *Sargassum plagiophyllum* (Phaeophyceae) were shown to have anti-cancer activity against HepG2 and A549 cell lines [45]. Porphyrans from *Porphyra* species induced cell death in human AGS gastric cancer cells in a dose dependent manner by decreasing cell proliferation and inducing apoptosis [46]. Carrageenan extracted from *Solieria chordalis* (Rhodophyta) showed no cytotoxicity towards human cancer cells lines but demonstrated immune-stimulating properties. Treatment resulted in enhancement of neutrophil phagocytosis, cytotoxicity by natural killer cells, antibody-dependent cell cytotoxicity and stimulation of lymphocyte proliferation, which points towards a use in cancer immunotherapeutic treatment [47]. A heterofucan isolated from *Spatoglossum schröederi* (Phaeophyceae), Fucan B, was found to inhibit the proliferation and migration of CHO-K1 when fibronectin was used as the substrate. Fucan B also promoted G_1 cell cycle arrest [30]. A summary of recently reported biological activities found in algal polysaccharides are outlined in Table 1.1.

Table 1.1 A wide variety of biological activities have been reported from algal polysaccharides. This table outlines observed activities in some recent studies.

Algal Polysaccharide	Extraction Method	Seaweed	Reported Activity	Ref.
Fucoidan	Hot water extraction	*Sargassum sglaucescens* (Phaeophyceae)	Anti-oxidant	[48]
Fucoidan	n/a	*Sargassum fusiforme* (Phaeophyceae)	Cognitive protective	[49]
Fucoidan	Ethanol extraction	*Isostichopus badionotus* (Stichopodidae)	Anti-inflammatory	[50]
Fucoidan	Ethanol extraction	*S. fusiforme* (Phaeophyceae)	Anti-angiogenic	[51]
Fucoidan	Ethanol extraction	*Coccophora langsdorfii* (Phaeophyceae)	Anti-cancer	[52]
Fucoidan	Methanol extraction	*Sargassum swartzii* (Phaeophyceae)	Anti-viral	[53]
Fucoidan	Ethanol extraction	*Fucus vesiculosus* (Phaeophyceae)	Anti-hyperglycemic	[54]
Fucoidan	n/a	*F. vesiculosus, Ascophyllum nodosum*	Anti-diabetic	[31]
Laminarin	n/a	*n/a*	Anti-fungal	[55]
Laminarin, Fucoidan	n/a	*Laminaria digitata* (Phaeophyceae)	Anti-oxidant	[56]
Agar, alginates	n/a	*Gelidium sp., Gracilaria sp., and A. nodosum*	Prebiotic	[18]
Alginic acid	n/a	*n/a*	Anti-oxidant	[57]
Alginate	n/a	*Durvillaea sp., Lessonia nigrescens* (Phaeophyceae)	Anti-obesity	[58]

1.3.2 Fatty Acids.

Fatty acids are commonly found in foods such as vegetable oils, meat, milk, and soy products. They play an important role in maintaining normal physiological functions. Docosahexaenoic acid (DHA) and arachidonic acid (AA) are both important parts of mammalian cell membranes and are crucial to brain and eye development in human infants. The intakes of omega-3 and omega-6 fatty acids have been linked to a reduction in cardiovascular mortality rates, suppression of arthritis-associated inflammation, and a decreased risk of cancer. Marine algae such as seaweed are a rich source of unsaturated fatty acids. An isolated diketosteroid, (*E*)-stigmasta-24(28)-en-3,6-dione (Compound 1) along with three previously known steroids from *Tydemania expeditionis* (Chlorophyta), namely β-sitosterol (2), fucosterol (3) and saringosterol (4) collected from the China sea were evaluated for activity on prostate cancer cell lines DU145, PC3 and LNCaP. The diketosteroid termed (compound 1), showed moderate inhibitory activities while fucosterol proved to be most effective. Two unsaturated fatty acids isolated from a Fijian population of the *T. expeditionis*, were shown to have moderate inhibitory activity against a panel of tumour cell lines (including breast, colon, lung, prostrate and ovarian cells [59].

1.3.3 Carotenoids and Terpenes.

Carotenoids are natural tetraterpenes which are produced by a wide variety of organisms ranging from single celled microbes to plants with more than 700 examples described so far [60]. The carotenoid β-carotene, which is found in large quantities in green and yellow fruit, and lycopene are both known for their anti-cancer activities [61]. Fucoxanthin is a carotenoid that is found in great abundance in Brown seaweeds [62]. Indeed, it is the most abundant of all carotenoids, accounting for more than 10% (approximately 10 million tonnes) of the estimated natural production of carotenoids each year [63]. Fucoxanthin is reported to be very effective in inducing cellular death in human leukaemia and colon cancer cells [64] and has been proven to suppress *in vivo* liver and skin carcinogenesis [61]. The ability to scavenge free radicals is thought to play an important role in the anti-mutagenic and anti-carcinogenic mechanisms of carotenoids, and as such, fucoxanthin displays potent scavenging abilities. To date, literature pertaining to the anti-cancer activity of carotenoids in seaweeds has focused

mainly on that of fucoxanthin. However, the exact mechanism by which fucoxanthin exerts it anti-cancer activity has not yet been fully defined. Fucoxanthin can strongly and concentration-dependently inhibit growth of human hepatoma cells and can facilitate growth of mouse embryonic cells. Fucoxanthin significantly enhanced gap junctional intercellular communication (GJIC) of the cancer cells without affecting noncancerous mouse cells. Treatment with fucoxanthin also resulted in an increase in both protein and mRNA expression. The upregulation of GJIC, coupled with increases in intracellular calcium levels may be responsible for cell cycle arrest and cellular death via apoptosis [63]. Fucoxanthin derived from seaweed *Undaria pinnatifida* (Phaeophyceae) was shown to markedly reduce the viability of different colon cancer cell lines *in vitro*. Treatment induced DNA fragmentation and reduced the level of the anti-apoptotic protein, Bcl-2. It was also noted that separate treatment of CaCo-2 cells with fucoxanthin and troglitazone recorded no decrease in cell viability, but when used in combination, cell viability was greatly reduced [62]. Fucoxanthin was shown to inhibit tumour cell growth in HepG2 cells by inducing G1 cell cycle arrest and/or inducing apoptosis [65]. In nature, most carotenoids occur predominately or entirely in the trans-form. The presence of a cis double bond garners greater steric hindrance between close-by hydrogen atoms and/or methyl groups, resulting in a bond that is less thermodynamically stable than the trans-form. The all-trans form of fucoxanthin was the major geometrical form found in the sample investigated. However, a mixture of 13-*cis* and 13′-*cis* isomers produced the strongest anti-proliferative activity of all the geometrical isomers [64]. Fucoxanthin from *Saccharina japonica* (formerly *Laminaria japonica*) (Phaeophyceae) has been shown to suppress the invasion of highly metastatic B16-F10 melanoma cells. This form of fucoxanthin inhibited the expression and secretion of MMP-9, which plays a critical role in tumour invasion and migration. Furthermore, the expression of cell surface glycoproteins that play an important role in migration, invasion and cancer-endothelial cell adhesion was diminished. In lung cancer metastasis models, fucoxanthin caused a significant reduction of tumour nodules [66].

Another carotenoid of interest is siphonaxanthin, which is a keto-carotenoid found in siphonaceous green algae. In comparison with other carotenoids such as fucoxanthin, siphonaxanthin is a potent inhibitor of HL-60 cells. Treatment with siphonaxanthin resulted in a significant reduction in cell viability within 6 h. An

increase in TUNEL-positive cells and chromatin condensation in the HL-60 cells indicated apoptotic activity. The induction of apoptosis also reduced the expression of Bcl-2 and increased the expression of caspase-3 [67]. Halogenated monoterpenes are produced by marine algae of the families Plocamiaceae and Rhizophyllidaceae and have a well-established anticancer potential. Four halogenated monoterpenes isolated from *Plocamium suhrii* (Rhodophyta) exhibited greater cytotoxicity when compared to cisplatin, a known anticancer drug, when assayed against an esophageal cancer cell line [68]. Polyhalogenated monoterpenes from *Plocamium corallorhiza* (Rhodophyta) also showed moderate cytotoxicity towards esophageal cancer cells [69] Peyssonoic acids A-B and a novel sesquiterpene hydroquinnones isolated from *Peyssonnelia* sp. exhibited modest antiproliferative activity against ovarian cancer cells [70].

1.3.4 Seaweed Derived Antioxidants.

Reactive oxygen species (ROS) are highly reactive molecules that are constantly produced by cellular enzymatic reactions. They are required to maintain cell homeostasis and the body's antioxidant defense systems are designed to prevent harmful effects caused by increased levels of ROS. Cells in a normal healthy condition produce ROS at low levels. Free radical-mediated modification of DNA, proteins, lipids and small cellular molecules have been associated with such diseases as cancer, atherosclerosis and rheumatoid arthritis [71]. Antioxidants are secondary metabolites that scavenge ROS and free radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxides and quenching superoxide and singlet oxygen [72]. Among marine organisms, seaweeds represent one of the richest sources of antioxidants [73]. In South East Asia, *Eucheuma cottoni* (Rhodophyta) is grown in abundance for human nutrition. A polyphenol rich extract from *E. cottoni* was shown to be anti-proliferative against oestrogen-dependent MCF-7 and oestrogen-independent MB-MDA-231 human breast-cancer cells *in vitro*, but non-toxic to non-cancerous cell lines. The extract was fed to female rats and following four weeks of dietary supplementation, mammary tumours were induced with carcinogenic agents. Tumour development and erythrocyte lipid peroxidation was inhibited in rats that had previously received the extract as well as induction of mammary tumour apoptosis,

down-regulation of oestrogen biosynthesis and an improved antioxidant status [74]. Soluble fractions of *Palmaria palmata* (Rhodophyta), *Laminaria setchellii* (Phaeophyceae), *Macrocystis integrifolia* (Phaeophyceae) and *Nereocystis leutkeana* (Phaeophyceae) have been shown to inhibit the proliferation of HeLa cells. The anti-proliferative effect of the seaweed extracts was positively linked to their total phenolic content [15]. One of the key antioxidant defense mechanisms in the cell is the NF E2-related factor 2 (Nrf2)—antioxidant-response element (ARE) signaling pathway, which can be activated by a variety of small molecules. Fractionation of the edible seaweed *Ulva lactuca* (Chlorophyta) gave rise to multiple active fractions as measured by an ARE-luciferase reporter assay. A keto-type C18 fatty acid was shown to induce the expression of cytoprotective genes with its cellular activity requiring the presence of Nrf2 and PI3k function. Mice treated with a single dose of an *U. latuca* fraction that was enriched with the C18 fatty acid showed similar ARE-activating effects to those observed in *in vitro* studies. This observation could be due to the ability of the fraction to inhibit KEAP1-mediated Nrf2 ubiquitination and the subsequent accumulation and nuclear translocation of Nrf2. A significant increase in the transcript levels of *Nqo*1 was also found in other mouse tissues such as the brain, stomach and lung [75].

Fucoidan has also been shown to exhibit antioxidant activity. When different sulphated polysaccharides from the seaweed *Turbinaria conoides* (Phaeophyceae) were evaluated for antioxidant activity, fucoidan showed the highest antioxidant potential followed by alginic acid and laminarin, respectively [71]. The anti-cancer properties of *Laurencia obusta* (Rhodophyta) were correlated with its total phenolic and flavonoid contents [76]. Polyphenol rich extracts from *Ecklonia cava* (Phaeophyceae) have shown strong anti-cancer activities. One study demonstrated significant suppression ($p > 0.05$) of migration and invasion of A549 cells in a dose-dependent manner and down regulation of the matrix metalloproteinase (MMP)-2 activity, which is essential in the degradation of the extracellular matrix [77]. Another poly-phenolic rich fraction from *E. cava* exhibited strong selective cell proliferation inhibition on all cancer cell lines tested (CT-26, THP-1, B-16 and U-937), which was attributed to induced apoptosis in CT-26. The extract also demonstrated strong radical scavenging activity and reducing power and at 5 µg/mL was found to be comparable to butylated hydroxytoluene at the same concentration [78]. Also, phloroglucinol derivatives from *E. cava* inhibited MCF-7 human cancer cells proliferation apoptosis

triggered through NF-κB family and NF-κB dependent pathways [79]. Oxidative stress brought about by long term exposure to ultraviolet radiation from sunlight plays an important role in the development of skin cancer. Ultraviolet B radiation in particular (by having a longer wavelength 280–320 nm) is associated with a more harmful impact on the skin. Protective compounds against biotic factors such as UV radiation has been produced by *Undaria crenata* (Phaeophyceae), with ethanol extractions having demonstrated photoprotective activity against cell damage caused by exposure to UVB radiation in Human HaCaT keratinocytes. Analysis revealed a significant scavenging effect of the extract against superoxide anion and hydroxyl radical. UVB-induced apoptosis was reduced, resulting in recovery of cell viability. Treatment also decreased the level of UVB-induced oxidative stress to lipids, proteins, and DNA, as shown by a decrease in the level of 8-isoprostane, protein carbonylation and DNA tails [80].

1.3.5 Anti-Cancer Activity of Minor Seaweed Components.

It is well documented that major seaweed components such as fucoidan and fucoxanthin have effective anti-cancer properties. However, the importance of screening crude seaweed extracts should not be overlooked, as minor components may also harbor potent biological activities. The sporophyll of *U. pinnatifida* is considered to have lower utility value compared to other parts of the plant and is usually discarded as waste. An ethanol extract of the sporophyll was prepared and shown to reduce the viability of colorectal cancer HCT116 cells [81]. A novel glycoprotein isolated from *S. japonica* (formerly *L. japonica)* (LJGP) was found to have anti-proliferative effects on numerous cancer cell lines in a dose-dependent manner. LJGP treatment of HT-29 cancer cells caused them to display several apoptotic features such as DNA fragmentation, sub-G1 arrest, caspase-3 activation, and Poly (ADP-ribose) polymerase (PARP) degradation. It was also determined that LJGP-induced apoptosis led to the formation of a death-inducing signaling complex (DISC) of Fas, FADD and procapase-8. LJGP induced the reduction of mitochondrial membrane potential with the activation of the Bcl-2 family of proteins and caspase-9 [82]. The enzyme telomerase adds tandem arrays of TTAGGG repeats to the ends of telomeres. Telomerase activity is not usually detectable in normal cells, but high activity is found in most cancer cells. Thus, telomerase represents a promising target for cancer therapy

and much work has been performed on screening for telomerase inhibitors. Eitsuka *et al.*, (2004) [83] confirmed the inhibitory effect of sulfoquinvosyldiacylglycerol (SQDG), a glyceroglycolipid, from *Porphyra yezoensis* on human telomerase in a cell-free system, which acted in a dose-dependent manner. It was also confirmed that EPA, which is a component of SQDG, is a potent telomerase inhibitor.

Three pigments isolated from an extract of *Porphyra tenera* (Rhodophyta) (β-carotene, chlorophyll a and lutein) showed significant activity against mutagen-induced *umu* C gene expression. Combined treatment with the pigments showed an additive effect compared with single treatment with each pigment [84]. The same authors later studied the *in vivo* anti-carcinogenic activity of the seaweed *Ulva prolifera* (formerly *Enteromorpha prolifera*) (Chlorophyta) using an initiator (7, 12-dimethylbenz[*a*]anthracene) and promoter (12-*O*-tetradecanoylphorbol-13-acetate) model. The application of *U. prolifera* extract prior to initiator or promoter treatment caused a significant suppression of mouse skin tumourigenesis. The combined use of the extract before both treatments (with initiator and promoter) resulted in much stronger suppression against the same skin tumourigenesis. It was proposed that a chlorophyll-related compound, pheophytin-a was an antigenotoxic substance [85]. The anti-tumour effect of pepsin-digested *Caulerpa microphysa* extracts was demonstrated by their addition to HL-60 and WEHI-3 cell lines. Growth of both cell lines was significantly affected ($p < 0.05$) when incubated with the digested extract at concentrations of 25 µg/mL and above. A significant increase in DNA damage was also recorded at concentrations of 100 µg/mL and above in comparison with the control cells [86].

1.4 Potential of Seaweed Components to Alleviate Cardiovascular Disease.

Cardiovascular diseases (CVD), including heart disease and stroke are a diverse group of disorders that affect the mammalian circulatory system. Collectively, CVDs are the number one cause of human death worldwide. In 2008, 30% of all deaths were because of a CVD. Although many risk factors for CVDs are recognised, the most important are hypertension, hyperlipidemia, hyperglycemia and abdominal obesity [87]. The traditional Japanese diet, which is characterised by high consumption of fish, seaweed and other plant material and sodium, with an accompanying decrease in refined carbohydrates and animal fat has been associated with a reduced risk of mortality associated with CVD. Today, Japan enjoys one of the lowest rates of coronary heart disease of any country in the world [88].

1.4.1 Hypertension and Hyperlipidaemia.

Hypertension or high blood pressure is a major modifiable risk factor of cardiovascular disease. Known as the "silent killer", hypertension can be asymptomatic for years before the condition is diagnosed clinically [89]. Cases of hypertension are divided into those of essential, primary or idiopathic hypertension with essential hypertension accounting for 95% of all cases. Risk factors that contribute to the development of hypertension include differing concentrations of sodium and potassium in the body, obesity, resistance to insulin, high alcohol intake, low calcium intake, stress and ageing. Many of these factors, such as being obese and having a high alcohol intake are additive over time. Approximately 25% of the global adult population suffers from hypertension, with this percentage expected to reach 60% of the population by 2025 [90, 91]. The prevalence of high blood pressure increases as people age. Indeed, in developed countries, 65% of those aged between 65 and 74 are affected by hypertension. Diet and lifestyle modifications are most often used to lower blood pressure levels [92].

Potassium alginate is a major polysaccharide present in brown seaweeds. Alginates are known to bind sodium, potassium and calcium ions and decrease the absorption of sodium in the intestine resulting in reduced blood pressure. In this

regard, dried seaweed flakes containing potassium alginate could be used as a replacement for table salt for people with high blood pressure [91, 93]. An epidemiological study performed in 25 countries spanning 15 years concluded that changes in dietary patterns such as cutting back on salt, increasing the consumption of fish oil, soybean protein and dietary fibre (including from seaweed) could reduce the risk of suffering a stroke [94]. Research into the effect of sulphated polysaccharides from *S. japonica* (formerly *L. japonica)* on rats with induced vascular endothelial damage after a psychological stress (PS) showed that adrenalin metabolites in plasma were significantly lowered in rats after administration of the seaweed extract. It was shown that the polysaccharide extract had a vascular endothelial cell-protective effect in stressed rats [95]. A low-molecular weight alginate extracted from *L. japonica* was shown to decrease systolic blood pressure in hypertensive rats. Rats that had high blood pressure displayed increased systolic blood pressure, sodium excretion, serum sodium and potassium levels, circulating plasma volume (CPV) and plasma atrial aldosterone (ALD) compared to a control group of non-induced rats. Treatment with the alginate extract normalised the induced changes. Furthermore, forms of potassium that do not contain chloride might offer better cellular entry in exchange for sodium and augment anti-hypertension activity [91]. Wakame powder from *U. pinnatifida* (5% *w*/*w* in a diet) significantly delayed signs of stroke and the survival rate of salt loaded, spontaneously hypertensive stroke-prone (SHRSP) rats [96]. In an early trial attempting to decrease sodium intake and increase potassium intake, a group of middle-aged patients suffering from mild hypertension were given a seaweed preparation (potassium loaded, ion-exchanging, sodium—adsorbing and potassium releasing). After four weeks of dietary intervention, there was a significant decrease in the mean blood pressure of those taking 12 and 24 g/day of the preparation [97]. While hypertension is mainly associated with adults, many studies have tracked blood pressure from childhood to adulthood with some showing that the process of atherosclerosis begins in childhood. Thus, monitoring blood pressure from an early age and appropriate intervention is important in preventing the development of CVD in later life. A study undertaken amongst Japanese preschool children examined the effect that seaweed intake had on blood pressure levels. Seaweed intake was significantly negatively related to systolic blood pressure in girls and negatively

related to diastolic blood pressure in boys suggesting that seaweed as part of the diet might have beneficial effects on blood pressure among children [98].

Hyperlipidaemia is a major cause of CVDs by bringing about sustained endothelial dysfunction and vascular inflammation [99]. A diet of restructured pork enriched with *Himanthalia elongata* fed to rats reduced plasma cholesterol levels in test subjects that were supplemented with dietary cholesterol [100].

1.4.2 The Renin-Angiotensin System.

The renin-angiotensin system (RAS) is a major regulator of blood pressure and fluid homeostasis in the body. Disruption of the RAS system can lead to increased blood pressure and the development of cardiovascular disease, chronic kidney disease and diabetes [101]. The two key enzymes in the RAS system are renin and angiotensin converting enzyme 1 (ACE-1). The inhibition of ACE-1 is a favoured strategy in treating hypertension [102] and several synthetic ACE inhibitors (captopril, lisinopril, enalapril and fosinopril) are thus used for this reason in the treatment of hypertension [103] Despite their effectiveness, synthetic ACE inhibitors are responsible for a number of unpleasant side effects such as development of a cough, loss of taste, renal impairment, and angioneurotic oedema and as a consequence there has been a trend recently to explore and develop more natural inhibitors of ACE activity [89]. Several studies have investigated the ACE inhibitory potential of compounds isolated from *U. pinnatifida*. Administration of the *U. pinnatifida* peptide led to a significant decrease in blood pressure in spontaneously hypertensive rats [104, 105]. A cold-water protein extract derived from *Porphyra columbina* (Rhodophyta) has been shown to have antihypertensive properties (>35% of ACE inhibition) [106]. *C. microphysa* pepsin digested extracts were determined to have greater ACE inhibitory activity than extracts digested with Flavourzyme or Alcalase [86]. Using enzyme hydrolysis, highly functional antihypertensive peptides have been produced from *Porphyra yezoensis* (Rhodophyta) Peptides produced under optimal conditions (1.5% substrate, 5% alcalase, pH 9.0, temperature of 50 °C and hydrolysis time of 60 min) had high antihypertensive activity (55% of ACE inhibition and a low IC_{50} value of 1.6 g/L [107]. A protein hydrolysate from *P. palmata* with *in vitro* renin inhibitory properties baked in bread was found not to have affected the texture or sensory properties of the

bread to a large degree. The bread containing the hydrolysate also retained the renin inhibitory activity following preparation and could represent a new method for the delivery of renin inhibitory substances [102].

1.4.3 Heart Disease.

Heart attack (myocardial infarction) is the leading cause of death for both sexes across the globe. It occurs when there is an insufficient blood supply to the myocardium leading to death of the myocardial muscle (ischemia). Prolonged ischemia leads to necrosis which is also termed myocardial infarction. Fucoidan from *C. okamuranus* was evaluated in rats where myocardial infarction was induced by isoproterenol, a synthetic catecholamine that has been known to cause severe stress in the myocardium. Fucoidan reduced the induced myocardial damage and improved the antioxidant defense system, reducing oxidative stress [108]. Similarly the pre-treatment of isoproterenol induced myocardial injured rats with fucoidan from *T. conoides* saw a significant normalization of the endogenous and exogenous antioxidant defense system [109]. Heparin is a widely-used anticoagulant that has unfortunate side effects such as bleeding and low platelet count (thrombocytopenia) as well as being potentially contaminated with prions and viruses from their animal sources. Fucoidans from algal sources are known to have anticoagulant activities and have been proposed as an alternative therapeutic treatment. Low and high molecular weight fucoidans were tested for their anti-aggregant, anti-coagulation and anti-thrombotic activities. When tested in the platelets of humans and rats, the high molecular-weight fucoidan showed pro-aggregation activity, whereas the low molecular-weight fucoidan demonstrated an inhibitory effect on thrombin induced aggregation, with an IC_{50} of 8 µg/mL, five-fold less than that of commercially available fucoidan or heparin. The inhibitory effects of low molecular-weight fucoidan and heparin on thrombin activity were greatly enhanced by either antithrombin or heparin cofactor II (HCII). Results indicated that low molecular weight fucoidan inhibits thrombin via activation of antithrombin and HCII, whereas commercial fucoidan mainly interacts directly with thrombin [110]. Other low molecular weight fractions of fucoidan from *S. japonica* (formerly *L. japonica)* were reported to also have strong anticoagulant activities [111].

1.4.4 Marine Derived Oils and Fatty Acids.

Omega-3 oils are produced naturally by algae and phytoplankton which are then consumed by fish resulting in an accumulation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in their flesh [112]. Human populations with a high consumption of fish have an inverse relationship with coronary heart disease and breast cancer. This inverse relationship first came to light from epidemiological studies of Inuit and Japanese populations, both of whom have lower incidences of CVD and cancer. In their homelands, the traditional diet of both groups contained appreciable amounts of fish. As they migrate over time to other areas, they adopt local dietary patterns and the incidences of CVD and cancer among them increase to the level of the local native people [112]. Fish and marine derived oils such as those from seaweed are rich in the omega (ω)-3 oils, eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3). Nutritional compositional studies of *Laminaria* sp., *U. pinnatifida*, *Sargassum fusiforme* (formerly *Hizikia fusiformis*) (Phaeophyceae) and *Porphyra* varieties found that they contained high levels of these oils [113]. Essential fatty acids play an important role in many biological processes. Following absorption from the gut, fatty acids are incorporated into triglycerides, phospholipids and cholesterol esters. Phospholipids are needed for the formation of cell membranes in every cell in the body. Omega-3 oils are fatty acids that have a signature double bond at the third position from the methyl (omega) end of the molecule. Such fatty acids cannot be synthesised by humans as the required enzymes to introduce a double bond at the correct position are missing. Such oils must be ingested as part of the diet [114]. The fluidity of the cell membrane is of great importance for receptor function and signaling pathways. The level of fluidity is determined in part by the amounts of phospholipids and fatty acids in the membrane that have double bonds. Multiple double bonds increase the fluidity of cell membrane and may partially account for the health benefits of omega-3 oils in preventing cardiac arrhythmias, as well as maintaining neurological function. DHA comprises only 4% of the fatty acid contents in the bloodstream but is almost 30% of the fatty acids in the phospholipids in the brain and retina, implying an important role in neurological and visual function [114]. In the mammalian heart, both DHA and EPA are incorporated into the cell membrane of cardiomyocytes, the levels of which can be significantly increased by taking food supplements containing omega-3 fatty acids. EPA and DHA,

released from myocardial membranes, exert anti-arrhythmic effects by prolonging the refractory periods of cardiac action potential. In a study of heart tissue from cadavers, levels of omega-3 and omega-6 fatty acids were found not to be associated with cardiac mortality. However, their presence in low levels (especially DHA and AA) were associated with high mortality in those with a history of coronary heart disease [115].

Ischemia-reperfusion injuries occur when tissues in the body are deprived of oxygen for a short period of time and the resumption of blood flow causes intense inflammation [116]. The intake of hydrogen gas has been shown to be an effective treatment for Ischemia-reperfusion injuries. Bacteria in the gut can produce hydrogen gas and it has been demonstrated that oral administration of mannitol to humans and animals can increase its production. Seaweed is a good source of mannitol and consumption can have a protective effect [117]. Seaweeds are also a good natural source of conjugated fatty acids (CFAs), isomers of PUFAs with a double bond in their structure. Dietary CFAs such as conjugated linoleic acids (CLAs) have been reported to prevent the onset of essential hypertension in non-obese hypertensive rats by regulating the production of physiologically active adipocytokines such as adiponectin, leptin and angiotensinogen [118].

1.5 Potential of Seaweed Components to Alleviate Diabetes Mellitus and Obesity.

Diabetes mellitus is a chronic disease where the pancreas does not produce enough insulin or when the body cannot use the insulin it produces effectively. Insulin is a hormone required for cells to take up glucose from the blood. People with diabetes exhibit an altered glucose metabolism [119] The inability to utilize glucose properly results in progressive complications in various bodily functions, and affects mineral levels in the body[120]. The vast majority of cases of diabetes mellitus present as either Type-1 diabetes or Type-2 diabetes, with Type-2 diabetes accounting for 90% of reported cases [121]. Since abnormally high or low blood glucose levels can lead to chronic cardiovascular problems, chronic renal failure, nerve damage, fainting and diabetic coma, people with diabetes must monitor and keep their condition under control at all times. Products obtained from nature have a long tradition of use in the treatment of diabetes. For example, plants that have high polyphenol content have the ability to inhibit the activity of carbohydrate hydrolysing enzymes such as α-amylase and α-glucosidase. This has the effect of lowering postprandial levels of glucose [122]. Seaweeds contain many components that are believed to be beneficial in the treatment of diabetes [123].

1.5.1 Seaweed-Derived Antioxidants in Treating *Diabetes Mellitus*.

In humans, a balance exists between the production of ROS and their inactivation as previously discussed. Oxidative stress comes about under certain conditions, such as *diabetes mellitus*, when the balance between production and inactivation is disrupted and ROS overwhelms the cell's antioxidant capabilities. Oxidative stress is reported to be the basal cause for the development of insulin resistance, β-cell dysfunction, impaired glucose intolerance and full blown type-2 diabetes [124, 125]. Several synthetic antioxidants, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are available commercially but their use is now being restricted due to adverse side effects, including the promotion of cancerous cells [126]. Because of this, interest in using naturally obtained antioxidants for diabetic treatment has increased [127]. Seaweeds are generally considered to be a

rich source of antioxidant compounds as previously discussed. Pigments such as fucoxanthin and astaxanthin, and polyphenolic compounds such as phenolic acid, flavonoid, and tannins have all exhibited antioxidant abilities [119]. Polyphenolic compounds can act as scavengers of ROS. However fundamental differences exist between the polyphenols produced by land plants and those produced by their marine counterpoints. This makes marine derived polyphenols a promising new target source for phenolic compounds that could be used as lead drugs in the pharmaceutical industry [124]. Edible seaweeds are a good source of polyphenols and by being able to modulate glucose-induced oxidative stress. Polyphenols are suggested to have anti-diabetic activity.

When extracting any potentially useful compound(s) from a source, it is important to remember that the profile of the extract is dependent on the profile of the solvent or solvents used. For example, ethanol and methanol can break down the plant cell walls more efficiently and are believed to be more effective at extracting antioxidant compounds from seaweeds than water [128]. The *T. conoides* showed higher total phenolic content (TPC) when developed as an extract in methanol, when compared to diethyl ether extract [129]. The brown seaweed *Tubinaria ornata* (Phaeophyceae) has demonstrated superoxide scavenging activity which may be effective in reducing the level of O_2 that is elevated during oxidative stress in the body. The presence of phenolic compounds suggests that the antioxidant activity might be due to them [130]. Methanol extracts from *E. cava* containing high levels of polyphenol and strong ROS scavenging ability significantly reduced blood glucose levels and increased insulin concentration when fed to type-1 diabetic rats. Blood alanine transaminase (ALT) levels were dramatically reduced to near normal levels. Increased levels of ALT in serum are often associated with health problems such as diabetes and liver damage. The anti-diabetic effect appears to be at least partly mediated by the activation of both the AMP-activated protein kinase/ACC and the Pl-3 kinase/Akt signal pathways [131]. The addition of edible seaweeds to foodstuffs may provide a useful method of enhancing their anti-diabetic qualities. Added *H. elongata, U. pinnatifida* and *Porhyra umbilicalis* (Rhodophyta) served as a source of soluble polyphenolic compounds in low salt meat emulsion model systems and increased the antioxidant capacity of the meat. The increased antioxidant load of the samples leads to greater stability during processing and storage of the meat [132]. Numerous other seaweeds have also been

found to contain high amounts of phenolic compounds and exhibited strong antioxidant activity, these include *Sargassum swartzii* (formerly *Sargassum wightii*) [126]; *Fucus serratus* (Phaeophyceae) and *F. vesiculosus* [133].

1.5.2 Controlling Glucose Levels in the Blood.

High levels of glucose in the blood following carbohydrate ingestion have an important role in the development of type-2 diabetes as well as in complications that rise from the disease. The control of postprandial hyperglycemia is of great importance in the treatment of diabetes and the prevention of cardiovascular complications. One avenue of treatment is to prevent the absorption of glucose by inhibiting carbohydrate-hydrolysing enzymes such as α-amylase and α-glucosidase. Synthetic inhibitors of α-amylase and α-glucosidase such as miglitol, voglibose and acarbose are designed to sharply reduce the blood sugar level that spikes after meals. The use of these however has several undesirable side effects such as flatulence, abdominal cramps, vomiting and diarrhoea. It has been suggested that adverse side effects are caused by excessive inhibition of pancreatic α-amylase and the delay of carbohydrate digestion. When undigested carbohydrates and other matter enters the colon, they give rise to increased bacterial fermentation and subsequently [134].

Seaweeds are known to have the ability to inhibit starch digestive enzymes and are an underexplored source of enzymatic inhibitors for use in the treatment of diabetes [135, 136]. A study investigating the α-amylase and α-glucosidase inhibitory effects of fifteen Irish seaweeds found that cold-water and ethanol extracts of *A. nodosum* had a strong α-amylase inhibitory effect while extracts of *F. vesiculosus* exhibited potent inhibition of α-glucosidase. The recorded effects of the extracts were associated with phenolic content and antioxidant activity [137]. The phenol rich extracts of *A. nodosum* collected from UK waters have also been shown to inhibit α-amylase activity to some extent. In a study conducted with samples of *A. nodosum*, *P. palmata* and *Alaria esculenta* (Phaeophyceae), the *A. nodosum* extracts were found to be the most active of the three seaweeds. The same extracts were also able to inhibit the activity of α-glucosidase at low levels. Following fractionation of the *A. nodosum* extracts, it was found that the inhibitory activity was concentrated in the phlorotannin rich fraction. It has been suggested that seaweeds accumulate phlorotannins to deter being

eaten by predatory species such as molluscs and they have been shown to potently inhibit the digestive glycosidases of marine snails [138]. Two bromophenols (2,4,6-tribromophenol and 2,4-dibromophenol) isolated and purified from the red seaweed *Grateloupia elliptica* (Rhodophyta) were found to have high α-glucosidase inhibitory activity. In addition, both compounds mildly inhibited rat-intestinal sucrase and rat-intestinal maltase. Both sucrase and maltase are similar in activity to α-glucosidase in so much as they break down sucrose and maltose to glucose. The authors of this study concluded that the bromophenols of *G. elliptica* have potential as natural nutraceuticals to manage diabetes mellitus [17]. Acetone crude extracts from *S. schroederi* and *Caulerpa racemose* (Chlorophyta) both inhibited α-amylase activity [139]. Dieckol isolated from *E. cava* showed pronounced α-amylase and α-glucosidase inhibition displaying higher activity than that of acarbose. Postprandial blood glucose levels in streptozotocin induced diabetic mice were also seen to be significantly suppressed [136]. Diphlorethohydroxyycarmalol (DPHC) isolated from *Ishige okamurae* (Phaeophyceae) showed strong inhibition of α-amylase and α-glucosidase without having any toxic effects on human umbilical vein endothelial cells (HUVECs) at various concentrations. In induced diabetic mice, extracts of *Petalonia binghamiae* (Phaeophyceae) (PBE) have been demonstrated to have anti-diabetic properties. Treatment with extract resulted in reduced blood glucose levels in diabetic mice and there was an improved tolerance to glucose [140]. Ethanol extracts from *Ulva rigida* (Chlorophyta) have been shown to have strong anti-hyperglycemic and antigenotoxic effects in diabetic mice [141].

1.5.3 Other Anti-Diabetic Activities.

Numerous studies indicate that a diet enriched with whole, unprocessed plant foods that are abundant in phytochemicals may be of benefit for metabolic disorders such as diabetes. Obese mice supplemented with an *U. pinnatifida* ethanol extract showed significantly reduced amount of visceral fat, adipocyte size, fasting blood glucose concentration and plasma insulin after nine weeks compared to the high fat fed control group. Results indicated that insulin resistance and hepatic fat build-up can be prevented by modulating the hepatic glucose and lipid homeostasis in the high fat induced obese mice [142]. Mice fed extracts of *I. okamurae* for six weeks were found

to have an improved blood glucose level and a lower level of blood glycosylated haemoglobin when compared to non-diabetic control mice. Data suggested that the *I. okamurae* extract lowered blood glucose levels by altering the activity of enzymes involved in glucose metabolism in the liver and by improving insulin resistance [143]. Dietary fibre present in seaweed such as alginates may reduce glycemic disturbances associated with obesity when included in the diet. A study involving forty self-reporting healthy males looked at the glycemic response to a controlled test lunch of varied composition following ingestion of an ionic-gelling alginate drink. It was seen that the alginate drink was able to attenuate the glycemic response following consumption of the test lunch [144].

One severe consequence of diabetes is the development of hyperglycemia-induced diabetic retinopathy (DR), a prevalent cause of blindness in many countries. LMWF from brown algae is known to demonstrate multiple biological activities (anti-inflammation, anti-oxidation, and anti-aggregation) which could be of benefit in treating ischemic disorders such as diabetic retinopathy. Calcium dobesilate is a strong antioxidant that is a current treatment for this condition. Mice with streptozotocin-induced diabetes were fed a diet containing LMWF (50, 100, or 200 mg/kg/day) or calcium dobesilate (50, 100, or, 200 mg/kg/day) for four months to examine the protective role of the LMWF against the development of diabetic retinopathy, the production of high glucose-promoted vascular endothelial growth factor (VEGF) and the proliferation of cells in microvascular endothelial cells. The LMWF alleviated retinal pathological change and hindered neo-vascularization due to diabetes *in vitro* [145].

1.5.5 Obesity.

Obesity is considered the gateway condition for several chronic diseases and is a major factor in the development of high blood pressure, type-2 diabetes, cardiovascular disease, and several types of cancer [146]. Obesity in children has been described as the most important health challenge of the 21st century, with the concern being that those individuals that are obese during their youth are likely to remain obese through to their adult life and as a result are more likely to develop cardiovascular diseases, cancer and diabetes [147]. One avenue of treatment is to manipulate the appetite and

reduce the amount of food and calories consumed. A reduction in casual snacking between meals and in portion size would have a major impact on obesity levels [148]. Satiety is an important factor in regulating the amount of food that people consume and has a great importance in public health as a means of controlling obesity. Satiety or the feeling of fullness implies that there is a cessation of hunger as a consequence of consuming food. This is due to many factors including energy density, weight and volume, macronutrient composition, particle size, appearance of the food, satisfaction upon eating it and palatability [149]. Dietary alginates can slow down the rate that nutrients are absorbed into the gut and promote satiety both of which are of consequence in controlling obesity and type-2 diabetes. Following ingestion, alginate formulations react with gastric acid and undergo ionic gelation in the stomach to produce a gel that can reduce the rate of gastric emptying, stimulate gastric stretch receptors, reduce the uptake of nutrients and influence the glycaemic response after meals [150]. A human intervention study, investigating different alginate solutions intended for use as dietary supplements to enhance satiety and limit energy intake in humans, found that consumption of a formulation with a low ratio of mannuronic acid to guluronic acid resulted in a decrease in self-perceived capacity for food intake and increased sensation of fullness [151]. Researchers in Korea found that the intake of oily foods and seaweed were among the factors associated with a higher risk of developing metabolic syndrome. However, data from animal studies have suggested that seaweed intake may be protective against weight gain. Another study by Maeda and colleagues found that mice fed a diet containing fucoxanthin resulted in significantly reduced ($p>0.05$) levels of abdominal white adipose tissue, blood glucose levels and insulin concentration [152]. Obesity can be characterised by an excessive deposition of fat with functional and morphological changes in adipocytes. The cell line 3T3-L1 is a preadipocyte cell line used in the study of adipogenesis. It has been shown that fucoxanthin enhances differentiation at an early stage but subsequently inhibits differentiation at the intermediate and late stages. Fucoxanthin also inhibited the uptake of glucose in mature 3T3-L1 adipocytes by reducing the phosphorylation of IRS-1 [131]. Fucoidan from the sporophyll of *U. pinnatifida* was investigated for anti-obesity potential through the inhibition of cytokines associated with inflammation. The presence of fucoidan significantly suppressed proliferator—activated receptor γ, C/EBPα, and adipocyte protein 2 while decreasing the expression

of inflammatory-related genes in 3T3-L1 adipocytes during adipogenesis. Fucoidan also reduced the synthesis of ROS and the build-up of lipids in the cells [153]. *F. vesiculosus* has been claimed to be a useful agent for the management of obesity. *F. vesiculosus* contains large amounts of iodine which is believed to stimulate the thyroid gland and have a subsequent effect on the metabolic rate. The presence of high levels of dietary fibre, phytosterols and tetraterpenes are also important in helping obesity management [154].

1.6 Dietary Fibre, Seaweed Polysaccharides and Prebiotics.

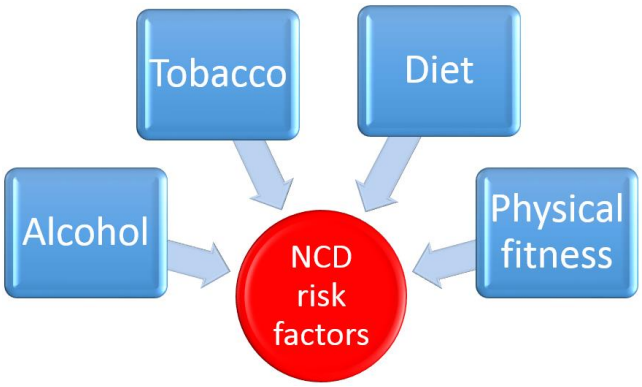
1.6.1 Dietary Fibre.

The main risk factors for NCDs for individuals are well known and are similar around the globe. Excess use of tobacco, harmful consumption of alcohol, low levels of physical activity and foods high in saturated trans fats, salts, and sugar account for two-thirds of all new cases of NCDs (Figure 5). In fact, the consumption of foods high in saturated and industrially produced trans fats, salt, and sugar is the cause of 40% of all deaths from NCDs [155]. A healthy diet coincides with lower incidence rates of CVD and other chronic diseases [156]. The dietary composition of humans has evolved greatly since the industrial revolution of the 18^{th} and 19^{th} centuries. Refined grains, meats, added fats and sugars have become more commonplace on the dinner table while the quantity of vegetables and fibre in our diets is reduced. This change in human nutrition, coupled with a more sedentary lifestyle is largely responsible for the increased level of obesity and other related chronic disease seen throughout the world today. Early humans had a predominantly plant based diet, similar to what modern apes live on today. This diet was high in fibre and low in sugar and, based on current dietary guidelines, would be expected to impart low serum cholesterol levels. Due to our close genetic relationship with modern apes, it is thought that the drastic changes in dietary habits that have taken place in the last two hundred years may help to explain our present day problems with chronic illnesses such as type 2-diabetes, obesity and heart disease [157]. Seaweeds are a good source of minerals and nutrients that are important for many biochemical reactions They are also rich in non-nutrient components such as dietary fibre and polyphenols [158]. Fibre is a generic term used to describe a broad family of carbohydrates found in plant cell walls. They are typically classified into three groups; soluble fibres (e.g., pectin and gums), insoluble fibres (e.g., cellulose) and mixed type fibre (e.g., brans). Generally, dietary fibres share the common characteristic that they are resistant to degradation by endogenous digestive tract enzymes, but can be broken down and fermented by the gut microbiota [159]. The definition of dietary fibre first appeared in 1953 in the context of describing the food components from plant cell walls. The current definition was suggested by the Codex Alimentarius Commission and defines DF as carbohydrate polymers with

ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belongs to the following categories [160].

The chemical and physical properties of dietary fibre largely dictate its physiological effects. Soluble dietary fibres swell in the stomach and increase the density of the stomach content, hindering the absorption of nutrients in the intestinal mucosa. This effect can be beneficial in controlling non-insulin dependent diabetes as it causes a decrease in blood glucose and insulin responses after meals. Also an increased sense of satiety or fullness after eating may be useful in the treatment of obesity and the prevention of obesity-linked chronic diseases [161]. Seaweed polysaccharides are mainly found in their cell wall where they confer strength and flexibility to the plant as well as maintaining the cells internal ionic balance preventing desiccation [162]. The complexity of seaweed structural polysaccharides, such as agar (red seaweeds) and alginates (brown seaweeds) make them resistant to degradation by human digestive enzymes and therefore available for fermentation by the gut microbiota in the colon. As such they can be regarded as a source of dietary fibre. The dietary fibre content of seaweed can range from 33% to 75% with the soluble fraction consisting of as much as 50%–80% of total dietary fibre content [18]. In general, seaweed polysaccharides are hydrophilic, often water soluble and are known to establish intra-chain hydrogen bond networks making them rigid and stiff and ideal for use as thickeners. Seaweed polysaccharides also promote interactions with external ions and inter chain hydrogen bonding making them useful as gelling agents [162]. Dietary fibre obtained from seaweed differs in composition, chemical structure, physio-chemical properties and biological effects from terrestrially derived fibre sources [163]. In this regard, structural polysaccharides from brown seaweeds, such as laminarin and fucoidan could offer a dietary means to modulate the gut microbiota (as in the case of prebiotics—discussed below) and/or modulate immunity [164].

Figure 1.5 The major modifiable risk factors for the development of a chronic non-communicable disease are (1) Alcohol intake (2) Tobacco (3) Diet (4) Physical fitness.



1.6.2 Prebiotics.

There has been an increase in interest over the last two decades in the adjustment of the composition of the gut microbiota to confer a health benefit upon the host (human or animal). One such area of research is of prebiotics. The prebiotic definition is constantly evolving as more information comes forth concerning the role of the gut microbiota in maintaining and promoting good health. The most recent definition of a dietary prebiotic is: "A substrate that is selectively utilized by host microorganisms conferring a health benefit" [165]. To be classified as prebiotic, several characteristics must first be met. The putative prebiotic must be able to resist digestion in the upper GIT, be selective in its stimulation of beneficial bacteria in the gut resulting in change in the profile of the microbiota and it must induce luminal or systemic effects that are beneficial to the health of the host [162]. Among the postulated health benefits of prebiotics for chronic conditions are: anti-colon cancer properties, osteoporosis management, improved bowel function, lipid lowering action, beneficial for cardiovascular disease associated with dyslipidemia and insulin resistance, obesity and possible type-2 diabetes. Seaweed derived polysaccharides (hydrocolloids) are potentially an important new source of prebiotics [166].

Bifidobacterium are well known for their ability to degrade complex carbohydrates in the colon and they are a common target for prebiotic ingredients. As a result, their genomes contain a relatively high number of genes (~8% of total genome) involved in the uptake of carbohydrates and metabolism, when compared to

other commensal bacterial genomes [167]. For a recent review on carbohydrate metabolism of the Bifidobacteria see Pokusaeva *et al*. [168]. Prebiotics have been found to be of benefit in chronic inflammatory bowel disease in transgenic rats by preventing the development of colitis. The protective effect was seen in association with an increase in the number of intestinal *Bifidobacterium* and *Lactobacillus* [169]. An investigation by [170] looked at the effects of supplying diets containing seaweed derived laminarin and fucoidan to growing pigs. During their weaning phase, pigs are susceptible to carrying *Salmonella typhimurium* and other pathogens. It was found that such a supplemented diet resulted in an increase in *Lactobacillus* numbers in the caecum and an increase of butyric acid in the caecum and colon. Increased shedding of faecal *S. typhimurium* at selected time points during the experiment was also recorded. A recent study by Ramnani *et al.* [18], investigated the prebiotic and fermentation potential of low molecular weight polysaccharides (LMWP) derived from agar and alginate using pH and temperature controlled anaerobic batch cultures that were inoculated with human faecal matter. Fluorescent *in-situ* hybridization (FISH) was used to monitor changes in microbial composition and gas chromatography was utilised to monitor the fermentation end products, short chain fatty acids. It was found that the LWMP derived from *Gelidium* spp. showed a significant increase in bifidobacteria populations from $\log_{10}$ 8.06 at 0 h to $\log_{10}$ 8.55 at 24 h.

Alginates from seaweed have also been used as an encapsulation agent for the delivery of probiotics. Alginates are non-toxic, biocompatible, inexpensive to obtain and are easily solubilised in the human intestine facilitating the release of their entrapped cells [171]. Magnesium is in abundant supply in fibre-rich foods such as seaweed. In animal trials, the intake of magnesium supplements prevented a drop-in resistance to insulin or glucose intolerance and postponed the development of spontaneous diabetes mellitus. Results from human trials show that dietary fibre or fibre rich foods can improve the after meal glycemic response, most likely due to lower rates of glucose absorption and increased utilization of glucose in the gut. The effect of magnesium and fibre intake on the development of diabetes in 1604 healthy subjects aged above 30 was assessed [172]. A total of 141 diabetes incidents were recorded during the follow-up period and it was concluded from the dietary intake information that lower levels of magnesium, lower total dietary fibre intake, or a combination of

both was associated with a higher risk of diabetes in the test population. The cell walls of some species of red seaweed contain the linear polymer agarose. The enzyme α-agarase can hydrolyse the α-1,3 linkage to produce agaro-oligosaccharide (AOS) while β-agarase works on the β-1,4 linkage yielding neoagaro-oligosaccharide (NAOS). NAOS was seen to be highly resistant to the enzymes of the upper GIT. NAOS significantly stimulated the growth of bifidobacteria and lactobacilli in MRS medium, compared with fructooligosaccharide (FOS), 1% (w/v) NAOS significantly promoted the specific growth rate of beneficial bacteria by approximately 100%. *In vivo,* NAOS significantly increased the numbers of lactobacilli and bifidobacteria ($p < 0.05$) in fresh feces or cecal content while reducing putrefactive microorganisms. NAOS with higher degrees of polymerization (DP) showed better prebiotic activity [173].

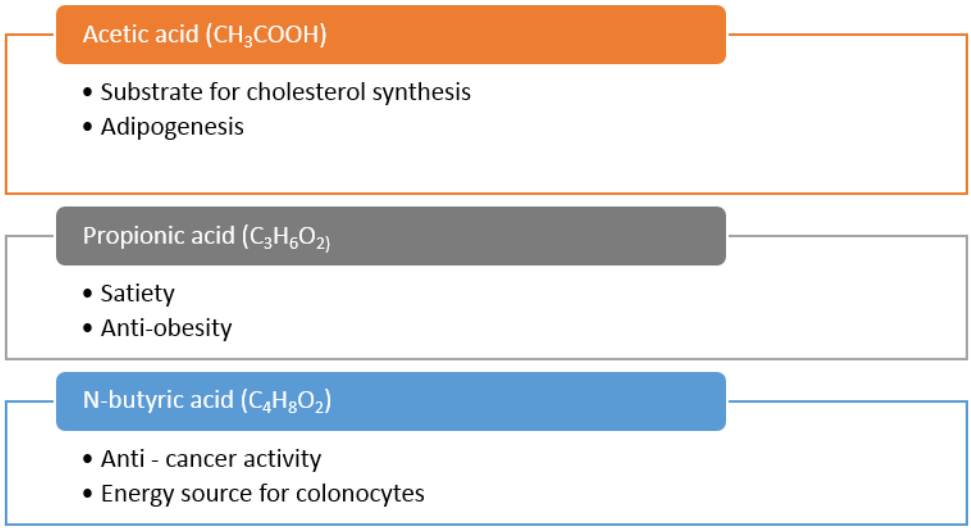
1.6.3 Production of Short Chain Fatty Acids (SCFA) by Colonic Bacteria and Health Benefits in Chronic Diseases.

Metabolites of bacterial metabolism are affected by the different types of food that we eat and by the subsequent production of bacterial enzymes such as β-glucuronidase, β-glucosidase, mucinase and urease. Through their action, the intestinal lumen can be exposed to detrimental toxic, carcinogenic or mutagenic substances. By changing the substrates that are made available in the gut, and favouring the production of beneficial metabolites such as short chain fatty acids (SCFA), a healthier environment can be established [174]. For example, the anaerobic microbial communities that inhabit the mammalian gastrointestinal tract produce SCFA (acetic acid (acetate), butyric acid (butyrate) and propionic acid (propionate)) as their main non-gaseous dietary fibre fermentation end products [175]. SCFAs can impart several health benefits on the host and the intake of seaweed can alter the SCFA production profile of the microbiota (Figure 6). Propionate has been shown to: lower the fatty acid content in the liver and in plasma, reduce the amount of food eaten at meals, demonstrate immunosuppressive activity and help tissue sensitivity to insulin, all of which can be of benefit in the treatment and prevention of obesity and type-2 diabetes [176]. Butyric acid is a preferred substrate for colonocytes and appears to promote a normal phenotype in these cells [177]. Butyrate has also received much attention as a chemoprotective agent

for colorectal cancer[178] while acetate has been shown to increase colonic blood flow and enhance ileal motility [179].

When healthy Wister rats were fed the red seaweed *Mastocarpus stellatus* (Rhodophyta) (10% algal supplemented diet) an increase in the molar concentration of both acetate and propionate was seen while butyrate molar concentrations decreased. There was also a decrease in the total levels of SCFA produced between the algal treated group and the basal diet control group as well as an increase in caecal pH [180]. Work has been carried out to evaluate the effect of seaweed derived laminarin and fucoidan on different indices of GIT fermentation in newly weaned pigs. Regarding the production of the three main SCFAs, the addition of laminarin to pigs' diet led to a significant increase in the concentration of acetic acid and a significant decrease in that of propionic acid in the caecum. The addition of fucoidan to their diet significantly increased acetic acid concentration and decreased the concentration of propionic acid in the same regions. Fucoidan also significantly increased butyric acid concentrations in both the caecum and the colon [170]. A study involving the *in vitro* fermentation of ten LMWP derived from agar and alginate from seaweed (*Gracilaria* spp. *Gelidium corneum* (formerly *G. sesquipedale*) (Rhodophyta) and *A. nodosum*) showed that the LMWPs caused a significant increase in total SCFA levels, especially acetic acid and propionic acid showing that they were readily fermented by the faecal bacteria [18].

Figure 1.6 Putative health benefits of the main short chain fatty acids (SCFAs), acetic acid, propionic acid and N-butyric acid. SCFA are mainly produced by the endogenous gut microbiota through the fermentation of undigested dietary fibres from the diet.



1.6.4 Potential of Seaweed Components to Alleviate Respiratory Diseases and Allergies.

Asthma is a complex inflammatory disease of the lungs characterised by variable airflow obstruction, airway hyper-responsiveness (AHR), and airway inflammation. The inflammatory response is characterised by infiltration of the airway wall by mast cells, lymphocytes and eosinophils and is associated with several inflammatory proteins, including cytokines, enzyme and adhesion molecules in the airway [181]. It is estimated that some 235 million people currently suffer from asthma, but it is likely that asthma is both under-diagnosed and undertreated worldwide. Asthma is the most common chronic disease affecting children. The fundamental cause of asthma is not completely known, but the use of medication and the avoidance of certain environments and triggers can reduce the severity of the condition. Triggers include indoor allergens such as dust mites, and pet dander; outdoor allergens like pollen and moulds; tobacco smoke; chemical irritants in the workplace and air pollution. Exposure to cold air and extreme emotional arousal (fear, anger) can also bring about attacks [182].

Polyphenolic extracts from the edible *Chondrophycus undulatus (*formerly known as *Laurencia undulata*) (Rhodophyta) have been shown to possess therapeutic potential for combating bronchial asthma associated with allergic diseases. Mice sensitised and challenged with ovalbumin (OVA) showed typical asthma symptoms as follows: an increase in the number of eosinophils in the bronchoalveolar lavage (BAL) fluid; a marked influx of inflammatory cells into the lung around blood vessels and airways, and airway luminal narrowing; airway hyper-responsiveness; detection of TNF-α and TH2 cytokines in the BAL fluid; and detection of allergen specific IgE in the serum. Intraperitoneal treatment of *L. undulata* extracts before the last airway OVA challenge resulted in significant inhibition of all asthmatic reactions [181]. Previously, extracts from the *E. cava* were also seen to be effective in relieving asthma symptoms in sensitised mice challenged with OVA by inhibiting the TH2 response [183].

Allergic diseases affect approximately one third of the general population. They are caused by chemical or immunological activation of mast cells leading to massive release of endogenous mediators such as histamine and a wide variety of inflammatory mediators such as eicosanoids, proteoglycans, proteases and several pro-inflammatory and chemotactic cytokines such as TNF-α, interleukin (IL-6, IL-4, IL-13). Preventing immune cells from degranulating is one of the crucial steps in preventing an allergic disorder. Two phlorotannins isolated from *E. cava*, 6,6′-bieckol and 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1, 4-dioxin were found to exhibit anti-allergic activities. The proposed mechanism of activity was that the seaweed compounds prevented degranulation by suppressing the binding of IgE and the FcεR receptor [184]. Allergic rhinitis (hay fever) is an inflammatory nasal disorder that involves activation and tissue recruitment of both structural cells and infiltrating leukocytes [185]. In a study to investigate a possible protective effect of the traditional Japanese diet on allergic disorders, it was observed that a high dietary intake of seaweed may be associated with a decreased prevalence of allergic rhinitis (hay fever) [186]. A high serum IgE concentration is a defining characteristic of atopic diseases such as atopic asthma and allergic rhinitis, with levels correlating with the extent and severity of the disease. Fucoidan from seaweed has been shown to reduce the increase of IgE in mice exposed to OVA. Fucoidan inhibited

IgE production by preventing the NFκB p52-mediated pathways activated by CD40 [187].

1.7 Conclusions.

Dealing with a chronic illness on a day to day basis is a fact of life for an ever-increasing proportion of the world's population. This is an unfortunate knock-on effect of extended human longevity and a decrease in incidence rates of infectious disease. The most important preventive measure one can take to avoid developing a chronic, non-communicable disease is to modify diet and lifestyle factors. It is well documented that excessive use of tobacco and alcoholic beverages, not being physically active or eating unhealthily will greatly increase an individual's chances of developing an NCD. However, for millions of people already suffering from chronic illness, simply avoiding risk factors is no longer an option. As the incidences of NCDs rise in the years to come, the burden on the world's health care services will also increase. New therapies will be sought out to provide better care and more economical services for long term patients. The emphasis up until now has been in searching the terrestrial regions of the planet for new drugs and bioactives. However, to face the new challenges of the future, new environments must be explored. The surface of the Earth is over 70% water and within the marine environments (the oceans, seas, rivers and lakes) of the planet, there exists an immense quantity of biological diversity with untapped potential. Seaweeds are a common sight along the coastal regions of the world. These marine plants have a long tradition of diverse use by mankind having been used for centuries in food preparation and traditional medicine. It is only in relatively recent times that the scientific community has developed the capabilities to better understand the health benefits of seaweeds that our ancestors knew of anecdotally. Indeed, the literature presented here clearly demonstrates the plethora of novel bioactives that seaweeds have to offer. While much of the research heralds the therapeutic effects from *in vitro* studies, the way has been laid to assess their efficacy *in vivo* through extensive animal trials and human clinical studies. In conclusion, there is little doubt that the fight to control the rise of chronic, NCDs will be a major challenge in the 21st century, but it is not an insurmountable one. The further education of current and future generations in making positive healthy lifestyle choices along with increased scientific research into the underlying causes of chronic disease and the development of new treatments can serve as a platform for effective future therapies.

1.8 References.

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Chapter 2

Screening and Analysis of Irish Seaweed Extracts for Bifidogenic Potential.

2.1 Abstract.

Seaweeds represent an underutilized reservoir of bioactive compounds that can be used in several diverse industries. They are highly bioactive, rich in non-digestible polysaccharides and have high fibre content. In addition, seaweeds have been suggested as a possible source of prebiotics. A prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit. They are resistant to hydrolysis by mammalian gut enzymes and pass through to the gastrointestinal tract intact until they reach the colon. Here, they become available to the colonic microbiota as fermentable substrates. Among the known beneficial bacterial species of the microbiota is *Bifidobacterium*, which is a major target for prebiotics. The aim of this study was to investigate the bifidogenic potential of fifteen extracts derived from Irish seaweed species using an *in vitro* anaerobic fermentation approach as follows. An initial screen comprising five different *Bifidobacterium* species (*B. breve* APC 325 NCBF 8807, *B. animalis* subspecies *lactis* Bb12 (APC 326), *B. longum* subspecies *longum* APC 422 DPC 6205, *B. bifidum* DPC 6034, *B. longum* subspecies *infantis* DPC 6036) representing the major *Bifidobacterium* species found in the mammalian gut, was used to test the ability of these beneficial microbes to utilise seaweed extracts as their sole carbon source for growth. In comparison with the control, an extract concentration of 2.5 mg/ml, eleven seaweeds (eight brown seaweeds and three red seaweeds) significantly ($p<0.05$) stimulated at least one of the *Bifidobacterium* strains at both time points (t= 20 h and 28 h), as measured through readings of optical density ($OD600_{nm}$). One extract, *Fucus serratus*, significantly stimulated the growth of four of the five *Bifidobacterium* strains at both time points. Further evaluation of the *F. serratus* extract at concentrations of 1.25 mg/ml and 0.625 mg/ml respectively also showed significant ($p<0.05$) stimulation of growth of *B. breve*, *B. lactis* and *B. longum*. Furthermore, appreciable levels of phenolic compounds were observed in several of the seaweed extractions including *F. serratus*. High-levels of such compounds are known to have antimicrobial activity and may explain an inhibitory effect observed with the *F. serratus* extract at a concentration of 2.5 mg/ml. The results of this study indicate that seaweed extracts have potential as good candidates for further prebiotic investigation using refined extraction and experimental methodologies.

2.2 Introduction.

Seaweeds belong to a rather ill-defined assemblage of marine plants known as algae. The term ‘seaweed’ itself does not have any taxonomic value, but is rather a popular term used to describe large attached (benthic) marine plants found in the groups that are referred to as the brown seaweeds (phylum Ochrophyta, class Phaeophyceae*)*, the red seaweed (phylum Rhodophyta) and the green seaweeds (phylum Chlorophyta). To date, about 6000 species of seaweeds have been identified. [2]. The brown and red seaweeds are almost exclusively located in marine environments while most green seaweed species are found in freshwater systems or on land. The predominant colouring of brown seaweed species derives from the dominance of the xanthophyll pigment fucoxanthin which masks other pigments that might be present. Red seaweeds are so coloured because of the presence of phycoerythrin, a pigment which absorbs blue light and reflects red, while the presence of chlorophyll *a* and *b* gives green seaweeds their distinctive colouring. Seaweeds are well regarded as a healthy food option as they are rich in polysaccharides, vitamins, minerals and polyunsaturated fatty acids (PUFAs) while also having a low lipid content [3]. Several countries of the Far East, especially China, Japan and Korea, have a strong tradition of seaweed consumption that stretches back several centuries. In the west, seaweed usage has mainly been limited to be a source of hydrocolloids. Recently however, an increasing number of people in western countries have begun consuming different seaweeds for their health benefits and nutritive value. Ireland boasts a remarkably high proportion of the marine algae found in the entire Atlantic basin, considering the size of the island. Ireland and Great Britain are in the temperate zone of the NE Atlantic which is an important biogeographic transitional area. The littoral and shallow sublittoral regions of these islands support over 650 seaweed species. This represents approximately 50% of the North Atlantics and 7% of the world’s documented seaweed species [4]. Brown seaweed is reported as a major marine bio-resource in the north Atlantic waters around Ireland and the UK. Fermentative biomethane production from seaweed via anaerobic digestion may be exploited soon as an attractive alternative to current energy sources. Ireland is one of the largest producers of seaweed in Europe with 13% of the total European harvest being collected here. This harvest in dominated by *Ascophyllum nodosum* (ca. 30,000 tonnes per year) and is mainly collected along the north-west coast of Ireland in Co. Donegal and Co. Galway [5].

The microbiota of a healthy individual is a highly diverse, and relatively stable community of microorganisms that is comprised of tens of trillions of members with thousands of species-level phylogenetic-types [6]. The human microbiota is rich at the species phylogenetic level, but somewhat limited in terms of the phylum level of diversity. The most commonly represented bacterial phyla in healthy adults are Firmicutes and Bacteroidetes, with significant numbers of Actinobacteria and Proteobacteria members also present [7]. Numerically, members of the microbiota outnumber the eukaryotic cells of the body by a factor of ten. Combined, they encode 3.3 million genes which code for a large spectrum of enzymatic activities in the gut environment including catabolic enzymes necessary for complex carbohydrate breakdown [8]. The microbiota is established after birth, with facultative anaerobic bacteria such as *E. coli* being among the first colonisers of the gut. As initial oxygen levels diminish, a dynamic anaerobic ecosystem is created in the gut where *Bifidobacterium* and other strictly anaerobic microbes such as *Clostridium* and *Bacteroides* dominate [9]. The genus *Bifidobacterium* is a member of the family Bifidobacteriaceae and the phylum Actinobacteria. They were first isolated from the faeces of a breast-fed infant by Tissier in 1899, and were then named *Bacillus bifidus [10]*. They are one of the main beneficial species of the microbiota and are among the most frequently used microorganisms in functional foods and dietary supplements. As such, they are major targets for both prebiotic and probiotic studies [11]. Bifidobacteria dominate the gut bacterial population in healthy breast-fed babies, although levels decrease following weaning. For adults, bifidobacterial population levels stabilise at around 3-6% of the total gut microbial population. As people age (>65 years), bifidobacterial numbers usually decline [12]. They are anaerobic, Gram-positive, non-motile, and nonsporulating irregular or branched fermentative rod bacteria. Traditionally, *Bifidobacterium* was considered a member of the lactic acid bacteria. However, as the mol% G+C content of its DNA is high and ranges between 55% and 64% it is now included in the Actinobacteria phylum [13]. Bifidobacteria have the ability to utilise different types of complex carbohydrates found in the diet that are not hydrolysed in the gut by the endogenous enzymes present [14].

A prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit [15]. Prebiotics have emerged as food ingredients with beneficial health promoting activity through stimulation of beneficial bacteria

(bifidobacteria/lactobacilli) and the production of the short-chain fatty acids, acetate, propionate and butyrate [16]. Prebiotics are commonly found in, or extracted from, fruits, cereal, and vegetables and are also present in human milk and colostrum. Candidate prebiotics include inulin-type fructans (ITF), fructooligosaccharide (FOS) and galactooligosaccharide (GOS). These compounds are predominately fermented in the proximal colon where the microbiota is known to have a saccharolytic metabolism [17]. It has been suggested that prebiotic intake may have numerous health benefits in humans such as: a reduction in the prevalence and duration of infectious and antibiotic-associated diarrhea; a reduction in inflammation and symptoms associated with inflammatory bowel disease; exertion of a protective effect to prevent colon cancer; the enhancement of bioavailability and uptake of minerals; a reduction of some risk factors for cardiovascular disease and the promotion of satiety, weight loss and the prevention of obesity [18]. Linear chained ITF prebiotics have consistently shown stimulatory effects on bifidobacterial populations along with an associated increase in saccharolytic fermentations resulting in SCFAs in both human and animal studies. [16]. The ability to utilize complex dietary and host glycans is central to the survival of prominent members of the gut microbiota. Dietary fruit, vegetables and cereals serve to provide readily digestible carbohydrates as well as dietary fibres (DFs) that resist both degradation and absorption in the small intestine. The bulk of DF consists of plant cell wall polysaccharides and resistant starch. These polysaccharides comprise structurally diverse sugar moieties linked by glycosidic bonds that form chains and branches. Remarkably, the human genome encodes, at most, only 17 enzymes for the digestion of food glycans, specifically starch, sucrose and lactose [7]. In comparison, more than 8% of the bifidobacterial genome is dedicated to carbohydrate metabolism [19]. Complex carbohydrates (oligo and polysaccharides) can both be degraded by several different enzymes produced by bifidobacteria. The fermenting activity of β-galactosidase, an example of a glycosyl hydrolase, is of high significance for bifidobacteria, as it ensures their growth in milk and dairy substrates containing β-galactoside bonds, including lactose [20].

Seaweeds have been linked with several positive effects in the human body, such as decreasing ammonia absorption, immune modulation, possible anti-metastatic activity, and increased short chain fatty acid (SCFA) production, and it has been suggested that the carbohydrates found in these plants are indeed the biologically

active components [17]. Seaweeds contain large quantities of complex carbohydrates that pass through the mammalian digestive system intact when ingested. Brown seaweeds mainly contain fucoidan, laminarin, cellulose, alginates and mannitol while red species of seaweed consist of starch, cellulose, xylan and mannan [21]. The cell walls of red seaweeds consists of polysaccharides such as agar, cellulose, xylan, mannan and carrageenan, while the cell walls of green seaweeds contain cellulose, mannose and xylan [22]. Different polysaccharides produced by seaweeds are used extensively in food, pharmaceuticals and other products for human consumption [23]. Most seaweed polysaccharides are non-digestible in the human gut given the absence of the required hydrolysing enzymes to break them down. As such, seaweed polysaccharides can be considered a source of DF and a potential source of novel prebiotics as they reach the colon intact where they may be fermented by the endogenous microbiota [24]. The seaweeds used in this study originated from along the Galway bay coastline, with two of the collection sites being in Co. Galway (Spiddal and Mweenish) and two in Co. Clare (Finnavara and New Quay). A great part of the Galway bay coastline is open to the full force of the Atlantic Ocean, especially in the northwest region where several of the bays are south-facing. Because of this south-facing direction, with relation to prevailing south-westerly winds and the coastal topography, rafts of seaweed uprooted by storms tend to be retained within the bay [25]. Irish coastal habitats vary considerably on spatial scales of kilometres with this being particularly true for the west coast of Ireland which has a highly indented topography [26]. These facts combine to make Ireland an ideal location for seaweed studies.

The aim of this study was to assess the bifidogenic potential of fifteen different cold-water extracts derived from a variety of Irish seaweeds, using an anaerobic *in vitro* assay. Pure cultures of different members of bifidobacterial species were assessed for their ability to ferment seaweed- derived extracts as their sole carbon source.

2.3 Materials and methods.

2.3.1 Seaweed abbreviations.

The different abbreviations used to denote the different seaweed species are as follows: AE - *Alaria esculenta,* AN - *Ascophyllum nodosum,* CC – *Chondrus crispus,* CF – *Codium fragile* FSE – *Fucus serratus,* FSP – *F. spiralis,* FV – *F. vesiculosus,* GG – *Gracilaria gracilis,* HE – *Himanthalia elongata,* LD – *Laminaria digitata,* LH – *L. hyperborea,* PC – *Pelvetia canaliculata,* PP – *Palmaria palmata,* SL – *Saccharina latissima,* UI – *Ulva intestinalis*.

2.3.2 Material.

All media and materials used in this study were obtained from Sigma-Aldrich, Dublin, Ireland unless otherwise stated.

2.3.3 Experimental Bifidobacterium strains.

All *Bifidobacterium* strains used in this study were obtained from the Teagasc Moorepark culture collection. The strains were as follows: *Bifidobacterium breve* APC 325 NCBF 8807 (*B. breve),* *Bifidobacterium animalis* subspecies *lactis* Bb12 APC 326 (*B. lactis*), *Bifidobacterium longum* subspecies *longum* APC 422 DPC 6205 (*B. longum),* *Bifidobacterium bifidum* DPC 6034 (*B. bifidum*), and *Bifidobacterium longum* subspecies *infantis* DPC 6036 *(B. infantis).* The strains were chosen to reflect the dominant species of *Bifidobacterium* found in the mammalian gastrointestinal tract, as well as the industrial important strain *B. lactis* Bb12. Each strain was grown on de Man, Rogosa, Sharpe (MRS) agar (Difco, Becton-Dickinson Ltd, Dublin, Ireland) supplemented with 0.05% (w/v) L-cysteine hydrochloride (MRS_{cys}). MRS_{cys} plates were incubated in an anaerobic jar (Merck KGaA, Darmstadt, Germany) with an AnaerocultA gas pack (Merck Millipore Ltd, Cork, Ireland) at 37 °C for 72 h. Stocks of all strains used in this study were stored at -80 °C with working stocks being stored at - 20 °C.

2.3.4 Experimental controls.

The control for the study was non-supplemented minimal media consisting of Tryptone (10 g/l), Yeast extract (2.5 g/l), Potassium phosphate dibasic (K_2HPO_4) 2 g/l, L-cysteine hydrochloride and Tween® 80 (1 ml/l). The known prebiotic fructooligosaccharide (FOS) from chicory was also include as a prebiotic reference. The FOS was obtained from Sigma-Aldrich. The positive control used in the growth study was the known prebiotic fructooligosaccharide (FOS) from chicory obtained from Sigma-Aldrich. The controls used in the *Fucus serratus* dose response assay was non-supplemented minimal media and FOS from chicory and.

2.3.5 Seaweed collection.

The cold-water extracts used in this study were all derived from different species of brown, red and green seaweeds. All crude seaweed materials were harvested between April 2009 and February 2011 at four collection sites in the Clare/Galway region of Ireland. These sites were Finnavara, Co. Clare (F), New Quay, Co. Clare (NQ), Spiddal, Co. Galway (S) and Mweenish Island, Co. Galway (M). The seaweeds species used in this study were as follows: *Alaria esculenta (*Linnaeus*)* Greville (S), *Ascophyllum nodosum* (Linnaeus) Le Jolis (NQ), *Chondrus crispus* Stackhouse (F), *Codium fragile* subsp. fragile (Suringar) Hariot (F) *Fucus serratus* Linnaeus (S), *Fucus spiralis* Linnaeus (S), *Fucus vesiculosus* Linnaeus (S), *Gracilaria gracilis* (Stackhouse) M. Steentoft, L.M.Irvine & W.F. Farnham (F) *Himanthalia elongata* (Linnaeus) S.F. Gray (F), *Laminaria digitata (Hudson)* J.V. Lamouroux (S), *Laminaria hyperborea* (Gunnerus) Foslie (M), *Pelvetia canaliculata* (Linnaeus) Decaisne & Thuret (S), and *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders (S) and Ulva intestinalis Linnaeus (S). As part of the Marine Functional Food Research Initiative, material from each of the collected seaweeds was kept in the National University of Ireland, Galway (NUIG) for reference.

2.3.6 Solid-liquid extractions.

Following their collection, each of the seaweed was washed in cold water to remove any attached particulate matter such as sand and then frozen immediately. All seaweed samples were freeze-dried and ground into a fine powder for storage in vacuum-packed bags at −80 °C. Solid liquid extractions, using cold-water as the solvent, were performed to extract bioactive compounds from the different seaweed samples. The seaweed extractions were performed in a large reaction vessel using an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, Ireland), with a ratio of seaweed material to cold-water of approximately 1:20. The reaction vessel was allowed to shake at 175 rpm for 3 h. The contents of the reaction vessel were then filtered through glass wool and concentrated using a rotary evaporator (Buchi Rotavapor R-220). Each extract obtained was freeze-dried to remove any remaining water and ground to a fine powder prior to storage at − 80 °C.

2.3.7 Bifidogenic screening of Irish seaweed species.

Each *Bifidobacterium* strain used in this study was routinely streaked out on de Man, Rogosa and Sharpe (MRS, Difco, Becton-Dickson ltd, Dublin Ireland) agar plates supplemented with 0.05% (w/v) L-cysteine hydrochloride (MRS_{cys}) and incubated anaerobically at 37 °C for 72 h. Single individual colonies were taken from the plates and cultured overnight in 5 ml MRS_{cys} broth and the following morning, the cell suspension was sub-cultured in 5 ml of fresh MRS_{cys} broth and allowed to reach an optical density (OD600nm) of 0.5 OD units. The extracts and controls were prepared in minimal media at an initial concentration of 2.5 mg/ml. Each extract was vortex-mixed vigorously for 5 min to aid their solubilization in the minimal media. The prepared extracts and controls were filter-sterilized using 0.45 µm filters prior to use. Filtered extracts were dispensed into clean, sterile universal containers in duplicate. Sub-cultured bacterial cells (1 ml) were centrifuged at 15,000 g for 5 min, and washed in minimal media twice prior to use. A 1% inoculum of washed cells was added to each tube and vortex-mixed for 10 sec. The tubes were then incubated anaerobically in a Whitley A85 anaerobic workstation (DW Scientific, Shipley, United Kingdom) at 37 °C. Readings of optical density were taken manually using a spectrophotometer at 0 h, 20 h, and 28 h**.** Further evaluation of an extract of interest was carried out at two

lower concentrations of 1.25 mg/ml and 0.625 mg/ml. Non-supplemented minimal media served as the control. FOS was used as positive a prebiotic reference for this analysis. As before, an extract concentration of 2.5 mg/ml was also included. The experimental procedure was as outlined above and carried out in duplicate. Readings of optical density were taken manually using a spectrophotometer at 0 h, 16 h, 20 h, 24 h and 28 h. Serial dilutions were carried out in maximum recovery diluent (MRD, Oxoid, Fisher Scientific, Dublin) with the appropriate dilutions being plated on MRS_{cys} agar plates. The agar plates were incubated for 72 h under anaerobic conditions at 37 °C. For each strain, a minimum of three biological repeats were carried out, n = 3.

2.3.8 Total carbohydrate and compositional analysis of seaweed extracts.

The total carbohydrate concentration of each seaweed extract was determined by using the resorcinol (1, 3 –dihydroxybenzene) sulphuric acid micromethod [27], with modifications. The method was scaled up with readings of OD being taken manually in microtubes using a spectrophotometer. Briefly, a 6 mg/ml resorcinol stock solution, a 14M H_2SO_4 solution, and a glucose control solution of 200 µg/ml were prepared using fresh deionised water. Each of the cold-water seaweed extracts was prepared at a concentration of 1 mg/ml in deionised water. The experimental conditions in each microtube were as follows: To each microtube, 200 µl sample, 200 µl resorcinol solution, and 1 ml of the H_2SO_4 solution were added. Each microtube was covered in tin foil and vortexed for 30 sec. The samples were then incubated at 90 °C for 30 min. After the allotted period, the samples were placed at room temperature and allowed to cool. Readings at an optical density of 430_{nm} and 480_{nm} were taken and the average value of total carbohydrates was obtained. The concentrations of the monosaccharides glucose and galactose were subsequently determined using an Aminex HPX 87C fixed ion resin column. The temperature of the column was set at 60 °C and the eluent 0.009N H_2SO_4 was run at 0.5 ml/min with. Glucose and galactose were detected using a refractive index detector. Each extract sample was filtered through a 0.22 µm microfilter before subjecting them to HPLC. Calibration curves for both sugars were generated using different concentrations (10, 20, 50, and 100 µg/ml) and gave a linear response [28]

2.3.9 Determination of total phenols content of Irish seaweeds.

The level of total phenolic compounds in the cold-water extracts was determined by using the Folin-Ciocalteu method and was externally calibrated using gallic acid. Briefly, 0.1 ml of extract solution (1 mg/ml), 0.1 ml of methanol and 0.1 ml of Folin-Ciocalteu reagent were added and the contents mixed thoroughly. After 4 min, 0.7 ml of 20% Na_2CO_3 was added, and then the mixture was allowed to stand for 30 min at normal temperature. The absorbance was then measured at 735 nm using a spectrophotometer. The concentration of total phenolic compounds was calculated as mg of gallic acid equivalents. The determination of total phenolic compounds in the fraction was carried out in triplicate and the results were averaged plus/minus standard deviation.

2.3.10 Statistical analysis.

This experiment was carried out at least in duplicate on three separate occasions. The results are presented as the mean value (± SE). Statistical analysis was carried out using GraphPad Prism version 5.01. An unpaired Student's t-test was used to evaluate significance. A p-value of <0.05 was deemed to be a significant result.

2.4 Results.

2.4.1 Carbohydrate analysis of seaweed extractions.

The total concentration of carbohydrates for each cold-water extract was determined using the resorcinol-sulphuric acid microplate method with modifications. Glucose was used as the control carbohydrate. The highest recorded total carbohydrate content of the ten brown seaweed species (Fig. 2.1) was observed with *S. latissima* (62.0 ± 0.7 GE mg^{-1}) followed by *F. serratus* (44.9 ± 2.5 GE mg^{-1}), and *A. nodosum* (44.6 ± 0.4 GE mg^{-1}). The recorded level of total carbohydrate content for the *Himanthalia elongata* cold-water extract was below the detectable range of the standard curve. Of the three red seaweed extracts (Fig. 2.1), the highest carbohydrate concentration was observed with *C. crispus* (49.2 ± 0.9 GE mg^{-1}), followed by *G. gracilis* (25.6 ± 0.6 GE mg^{-1}) and *P. palmata* (9.9 ± 0.1 GE mg^{-1}), while, of the two green seaweeds (Fig. 2.1) evaluated, the highest total carbohydrate content was recorded with *U. intestinalis* (21.8 ± 0.1 GE mg^{-1}) followed by *C. fragile* (2.2 ± 0.5 GE mg^{-1})

The ten brown seaweed extracts were also analysed for their free glucose, and galactose content (Fig. 2.2) using HPLC. Notable concentrations of glucose were found with *S. latissima* (42.6 µg/ml), *L. hyperborea* (28.7 µg/ml), *L. digitata* (11.8 µg/ml), and *A. esculenta* (7.3 µg/ml). No free glucose was observed with the *H. elongata, F. serratus, F. vesiculosus, P. canaliculata* extracts. No free galactose was present in any of the tested extracts.

2.4.2 Determination of Total Phenolic Content.

Total phenolic content was determined by using Folin-Ciocalteu reagent and external calibration with gallic acid (Fig 2.3) Of the brown seaweed extracts, the highest concentration of phenolic compounds was found in *F. vesiculosus* (132.5 ± 1.9 µg GAE mg^{-1}), *P. canaliculata* (107.0 ± 0.9 µg GAE mg^{-1}) and *A. nodosum* (89.3 ± 1.1 µg GAE mg^{-1}) (Fig 2.3 (a)). The highest concentration of phenolic compound found in extracts from red seaweed species (Fig 2.3 (b)) was that of *P. palmata* (8.4 ± 0.3 µg GAE mg^{-1}), followed by *C. crispus* (6.3 ± 0.3 µg GAE mg^{-1}) and *G. gracilis* (4.6 ± 0.1 µg GAE mg^{-1}). Of the green seaweed species (Fig 2.3 (c)), the concentration of

phenolic compounds was found highest in *U. intestinalis* (20.7 ± 0.6 GAE mg^{-1}) followed by *C. fragile* (0.8 ± 0.1 GAE mg^{-1}).

2.4.3 Bifidogenic potential screen of cold-water extracts from ten brown Irish seaweeds species.

Cold-water extracts at a concentration of 2.5 mg/ml from ten brown seaweeds were investigated for their bifidogenic potential using *B. breve, B. lactis, B. longum, B. bifidum* and *B. infantis* as indicator test strains. In comparison with the cellulose control, *A. esculenta, A. nodosum, C. crispus, C. fragile, F. serratus, G. gracilis, H. elongata, L. digitata, L. hyperborea, P. canaliculata,* and *U. intestinalis* all significantly increased ($p<0.05$) the relative OD at either 20 h or 28 h of at least one of the indicators strains. The greatest stimulatory activity observed amongst the brown seaweed extracts was observed for *F. serratus.* This extract significantly increased ($p>0.05$) the relative OD of four of the five indicator strains, *B. breve* (Fig 2.4), *B. lactis* (Fig. 2.6), *B. longum (*Fig 2.8), and *B. infantis* (Fig 2.12), at both time points (t = 20 h and 28 h), in comparison with the cellulose control. The second highest stimulatory activity was observed with the extract from *S. latissima*. This extract significantly increased ($p>0.05$) the relative optical density of three of the indicator strains, *B. breve* (Fig 2.4), *B. longum* (Fig. 2.6), *B. bifidum* (Fig 2.10), at both time points (t = 20 h and 28 h) in comparison with the cellulose control. The lowest overall level of stimulatory activity amongst the brown seaweeds was observed for the extract from *F. vesiculosus* and *A. esculenta*. The relative percentage change in OD_{600nm} for the brown seaweed extracts for *B. breve* (Table 2.1), *B. lactis* (Table 2.3), *B. longum (*Table 2.5), *B. bifidum* (Table 2.7) and *B. infantis* (Table 2.9) are detailed below.

2.4.4 Bifidogenic potential screen of cold-water extracts from red and green Irish seaweed species.

Cold-water extracts at a concentration of 2.5 mg/ml from three red seaweeds and two green seaweeds were assessed for prebiotic potential. The bacterial strains used were as previously described. The greatest stimulatory activity amongst the red seaweed extracts was observed for *G. gracilis.* This extract significantly increased ($p>0.05$) the relative OD of *B. longum* (Fig. 2.9) at both time points (t = 20 h and 28 h) and for *B. breve* (Fig 2.5) at 28 h only, in comparison with the cellulose negative control. For the green seaweed species, the extract from *U. intestinalis* significantly increased the growth ($p<0.05$) of *B. breve* (Fig 2.5) and *B. longum* (Fig. 2.9) at both time points (t = 20 h and 28 h) and the extract from *C. fragile* significantly increased the relative OD of *B. longum* (Fig 2.9) at 28 h only, in comparison with the cellulose control. Neither of the green seaweed extracts had a significant positive effect on the growth of *B. lactis*. The relative percentage change in OD_{600nm} for the red and green seaweed extracts for *B. breve* (Table 2.2), *B. lactis* (Table 2.4), *B. longum (*Table 2.6), *B. bifidum* (Table 2.8) and *B. infantis* (Table 2.10) are detailed in below.

2.4.5 Further analysis of the bifidogenic potential of the brown seaweed *F. serratus.*

Further investigation of the bifidogenic stimulatory effect of the *F. serratus* extract was undertaken at 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml. Readings of OD_{600nm} were manually taken at 16 h, 20 h, 24 h, and 28 h. The indicator test strains were *B. breve* (2.14 (a), *B. lacti*s (Fig.2.14 (b), and *B. longum* (Fig. 2.14 (c)). A notable inhibitory effect was observed for the *F. serratus* extract at a concentration of 2.5 mg/ml for *B. breve* and *B. lactis*. The greatest stimulatory effect of *F. serratus* extract was observed at a concentration of 1.25 mg/ml. At this concentration, the extract significantly increased ($p>0.05$) the relative OD of *B. breve, B. lactis* and *B. longum* at all time points (t = 16 h, 20 h, 24 h and 28 h), in comparison with the cellulose negative control (Fig. 2.14). Stimulation of *B. longum* by the *F. serratus* extract was significantly ($p>0.05$) increased over that observed with the FOS control at all time points (t= 16 h, 20 h, 24 h, and 28 h), and for *B. lactis* at 28 h only. The lowest bifidogenic stimulatory effect with the *F. serratus* extract was observed at a concentration of 0.625 mg/ml.

The greatest bifidobacterial stimulatory effect for the FOS control was observed at a concentration of 2.5 mg/ml. At this concentration, significant increases in relative OD were observed for *B. breve, B. lactis* and *B. longum* at all time points (t= 16 h, 20 h, 24 h, and 28 h). No inhibitory effect was observed with FOS. The relative percentage change in OD_{600nm} for different concentrations of the *F. serratus* extract and FOS for *B. breve, B. lactis*, *B. longum, B. bifidum,* and *B. infantis* are detailed in below (Table 2.11).

Serial dilutions were prepared in maximum recovery diluent at 24 h and plated on to MRS_{cys} plates to quantify the recovery of viable *Bifidobacterium* colony forming units (CFUs) (Fig. 2.15). Significant increases ($p<0.05$) in recoverable bacterial CFUs were observed with the *F. serratus* extract for *B. longum* at 0.625 mg/ml and 1.25 mg/ml but not at 2.5 mg/ml, in comparison with the negative control No significant changes in recoverable bacterial CFUs were observed with the *F. serratus* extract at this time-point for *B. breve* or for *B. lactis* at any concentration. A significant increase ($p<0.05$) in recoverable bacterial CFUs were observed with the FOS positive control for *B. longum* at a concentration of 0.625 mg/ml but not at 1.5 mg/ml or 2.5 mg/ml. These was no significant impact on recoverable CFUs with FOS for *B. breve* or *B. lactis.*

2.5 Discussion.

The main aim of this study was to ascertain whether different strains of bifidobacteria could utilize the complex assortment of monosaccharides, oligosaccharides and polysaccharides found in species of Irish brown, red and green seaweeds as their major energy source for growth. Improving host health, as well as reducing the threat of chronic disease, is among the driving forces for the development of functional foods for humans and animals. At present, there is both a scientific and commercial interest in the concept of prebiotics, which aims to beneficially modulate the composition of the gut microbiota and associated metabolic activities [19]. Any food ingredient that enters the large intestine has the potential to be a prebiotic compound and exhibit bifidogenic effects [29], however not all will. The genus *Bifidobacterium* is a major grouping of beneficial microorganisms and have been a major target for prebiotic compounds since the very beginning of prebiotic research. They are associated with many positive health benefits such as modulation of the intestinal barrier and of the immune response and the exclusion of pathogens [30]. The bifidobacteria used in this study are all prominent members of the healthy human gut microbiota. *B. breve* is the most common species of *Bifidobacterium* in breastfed and preterm infants and is the best-characterised of the bifidobacteria. This species appears to have an affinity for the immature bowel and colonisation is associated with fewer abnormal abdominal signs and better weight gain in very low birthweight infants [31, 32]. In 2006, *B. infantis* and *B. longum*, along with *Bifidobacterium suis* were unified into a single species, *B. longum*, based on DNA-DNA hybridization values. [33]. *B. longum* subspecies *longum* represents the most common subspecies [34]. *B. longum* subspecies *infantis* is unique for its ability to digest and consume many human milk oligosaccharides. This species of *Bifidobacterium* possesses a large repertoire of bacterial genes encoding for glycosidases and oligosaccharide transporters not found in other bacterial species. *B. infantis* grows better *in vitro* than any other bacterial strain in the presence of human milk oligosaccharides [35]. *Bifidobacterium animalis* subspecies *lactis* Bb12 (*B. lactis* Bb12) is a commercially available probiotic strain used throughout the world in a variety of functional foods and dietary supplements. The health benefits of *B. lactis* Bb12 have been well-documented in several independent clinical trials [36]. Members of the taxon *B. bifidum* have been shown to display remarkable physiological and genetic features involving adhesion to epithelia,

as well as host-derived glycans [10]. The human genome is predicted to encode just eight glycosyl hydrolases (GHs) that are directly linked to carbohydrate metabolism. Accordingly, many complex dietary carbohydrates remain undigested and end up in the colon intact where they can serve as sources of fermentable substrates for the microbiota [12]. Carbohydrate metabolism in *Bifidobacterium* is adapted for life in the mammalian GIT environment as seen from the presence of genes for GHs, sugar ABC transporters, and phospohenolpyruvate-phosphotransferase (PEP-PTS), all of which are required for the breakdown of plant and host-derived carbohydrates. A large percentage of the genes in a given bifidobacterial genome is predicted to be involved in sugar metabolism with half of these devoted to carbohydrate uptake [37]. Key to carbohydrate metabolism in the bifidobacteria is phosphoketolase (PK), a prominent thiamine diphosphate-dependent (TPP) enzyme in microbial sugar metabolism. This enzyme is central to the fructose-6-phosphate phosphoketolase (F6PK) of bifidobacteria [38, 39]. Bifidobacteria are thought to degrade hexoses exclusively by F6PK pathway, which is also termed the 'bifid' shunt [40]. PK can catalyse the formation of acetyl phosphate and erythrose-4-phosphate from fructose-6-phosphate, or the formation of acetyl phosphate and glyceraldehyde-3-phosphate from xylose-5-phosphate utilising inorganic phosphate as acceptor [39]. PK is a taxonomic marker for the family Bifidobacteriaceae.

With this study, the initial step in investigating the prebiotic potential of Irish seaweeds was taken by using a targeted bifidogenic screen. Screening consisted of a panel of fifteen cold-water extracts, prepared from different Irish seaweed species, being tested against five bifidobacterial strains obtained from an in-house culture collection. To grow and multiply successfully, microorganisms need the right assortment of nutrients, a source of energy that they can metabolise and certain environmental conditions conducive to their survival. In the laboratory, these requirements must be met by a culture medium. A defined media is one that contains a simple sugar as a source of carbon and energy, an inorganic source of nitrogen as well as mineral salts and any required growth factors. In contrast, a minimal media provides only the exact essential nutrients needed for a particular organism to grow [41]. The media used in this study was a specifically designed 'bifidogenic' minimal media, containing all the required nutrients to promote bifidobacterial growth except for a primary carbon source. The addition of the seaweed extractions to this media

would serve as the only major source of carbohydrates available to the bifidobacteria for growth. The primary control for the bifidogenic screen was minimal media without a supplementary source of carbon. A positive control, in the form of the known prebiotic FOS was also included. Growth/stimulatory activity was measured by taking optical density readings at a wavelength of 600 nm (OD_{600nm}) at three different time-points. The first step in accessing seaweed carbohydrates is to extract them in an inexpensive, environmentally friendly way. A variety of modern extraction methods, such as supercritical and subcritical-fluid extraction, microwave-assisted extraction and pressurised liquid extraction have been developed in recent years that improve on conventional extraction methods in terms of organic solvent consumption, extraction efficiency, selectivity and ease of automation for the extraction of natural compounds [42, 43]. Here, atmospheric pressure solid-liquid extraction was used for the extraction of bioactive components from the main biomass bulk. This method is relatively inexpensive in terms of capital costs and requires less state of the art knowledge and optimisation than other extraction methods. Solvents that can be used for extraction of seaweed bioactive compounds range from 100% water to mixtures of ethanol or acetone. The use of water as the solvent in previously reported extractions resulted in the highest yields. This reflects the hydrophilic nature of the majority of the components within seaweed and is in part explained by the high quantities of polysaccharides present [44].

The supplementation of the minimal media with different seaweed extracts was generally met with positive stimulatory/growth outcomes. As the only major source of carbohydrates available, it was clear that the respective bifidobacteria were successfully able to utilize the seaweed extracts. Eight out of the ten brown seaweed extracts (*F. serratus, S. latissima, L. hyperborea, P. canaliculata, A. nodosum, F. spiralis, H. elongata, and L. digitata)* and all red seaweed extracts significantly increased ($p<0.05$) the growth of one or more of the test strains, as compared to the negative control. As expected, this result strongly corresponded with the total concentration of carbohydrates in the seaweed extracts, which was determined using a modified resorcinol-sulphuric acid microplate method. None of the tested seaweed extracts had a significant effect on all five of the test strains. It is important to note that the beneficial effect on a given strain of *Bifidobacterium* is strain specific [45]. Brown, red and green seaweeds are all known to be rich in carbohydrates however

brown seaweeds are especially rich in soluble fibre [3]. The cell walls of brown seaweeds contain large amounts of polysaccharides such as laminarin, a β-polymer of glucose that represents the main storage polysaccharide in algae, and fucoidan, a sulphated heteropolysaccharide which is mainly composed of L-fucose, and mediates a protective effect against desiccation [46]. Ten of the fifteen seaweed extracts were found to have a total carbohydrate content in excess of 20 GE mg^{-1}. No free glucose or galactose were observed with the *F. serratus and F. vesiculosus* extract nor with the extracts from *H. elongata* and *P. canaliculata*. However, appreciable levels of free glucose were found with the *A. esculenta, L. digitata, L. hyperborea,* and *S. latissima* extracts. Higher levels of total carbohydrate in general mirrored observed bifidogenic affects. The most evident bifidogenic stimulatory effect amongst the extracts was observed with the *F. serratus* cold-water extract. Supplementation with this extract at a concentration of 2.5 mg/ml significantly stimulated ($P<0.05$) four out of five test *Bifidobacterium* strains (*B. breve*, *B. lactis*, *B. longum* and *B. infantis)* at both experimental time-points. As such, the *Fucus serratus* extract was chosen for further analysis of bifidogenic potential. Readings of optical density at additional time-points were made (16 h, 20 h, 24 h and 28 h) with the extract concentration ranging from 2.5 mg/ml to 0.625 mg/ml. The same positive and negative controls as the previous screen were used. An expected concentration dependent effect was observed with FOS for all three test strains, with the highest levels of stimulation being observed at a of 2.5 mg/ml and the lowest at 0.625 mg/ml. Interestingly, at a concentration of 2.5 mg/ml an inhibitory effect was observed with the *F. serratus* extract affecting all test strains, but most notably *B. breve* and *B. lactis.* A significant reduction in OD readings ($P<0.05$) was recorded with *B. lactis* at this concentration for all time points in comparison with the *F. serratus* extract at a concentration of 1.25 mg/ml. A similar decrease was apparent for *B. breve*; however, there was no significant difference between results at 2.5 mg/ml and 1.25 mg/ml. This observation was also evident for *B. longum*. We propose that an inhibitory substance, naturally occurring within the seaweed, was the causative agent of the observed inhibition of *Bifidobacterium* growth. The results obtained for both FOS and the *F. serratus* extract at 2.5 mg/ml were comparable to that observed during the initial bifidogenic screen for all test strains, indicating that the effect is reproducible *in vitro.* The data from both the initial screen and the subsequent dose response investigation underline the theory that any

putative bifidogenic stimulatory activity observed with the seaweed extracts is strain dependent. This fact is supported by the plate count data obtained for the *F. serratus* extract. Significant increases in CFU/ml were only recorded for *B. longum*. No significant changes were observed with either *B. breve* nor *B. lactis*. This agreed with the optical density readings obtained. Differences in observations between readings of optical density and plate counts could be as a result of pipetting/serial dilution errors Utilisation of FOS by the bifidobacteria was varied and strain specific. Significant increases ($P<0.05$) in CFU/ml at were only observed for *B. breve* and *B. longum* at the two lower concentrations and not at 2.5 mg/ml. Differences in the fermentative ability of the different *Bifidobacterium* strains to use seaweed carbohydrates, such as fucoidan and laminarin, suggest that variations exist in their genomes with respect to complex carbohydrate utilisation.

Dietary polyphenols are natural compounds occurring in plants and are characterized by hydroxylated phenyl moieties [47]. Polyphenols can affect human health because of their antioxidant and antimicrobial properties as well as free-radical scavenging activity [48]. A high proportion of polyphenols from our diet are not absorbed directly, so these compounds can directly reach the gut and modify the composition of the gut microbiota. Because of their ability to metabolise polyphenols, bacteria may play an important role in the production of new compounds *in situ*, which could have better bioavailability than their parent compounds. The transformation of these in the gut depends on microbial esterase and glucosidase as well as on demethylation, dehydroxylation and decarboxylation activities. In parallel to microbial catabolism, unabsorbed dietary polyphenols and their metabolites can behave as activators or inhibitors of bacterial growth depending on their chemical structure and concentration. These metabolites selectively inhibit pathogens and stimulate the growth of commensal bacteria, including some recognized probiotics [49]. A study carried out by Queipo-Ortuno et al. [50] showed that the daily consumption of red wine polyphenols significantly increased the numbers of several genera of beneficial bacteria including *Bifidobacterium*. There are many studies describing the influence of polyphenols on the growth and viability of lactic acid bacteria but mainly from the genus *Lactobacillus*. Gwiazdowska et al. [48] demonstrated that polyphenols exhibit both stimulatory and inhibitory effects on the growth of bifidobacteria. The inhibitory effect of polyphenols was most evident during

the first hours of incubation. Most studies highlight the fact that the effect of polyphenols depends on the type of polyphenol, its structure, its concentration and also the susceptibility of the bacterial strain in question. High levels of phenolic compounds (< 50 GAE mg^{-1}) were present in the brown seaweeds; *F. vesiculosus, P. canaliculata, A. nodosum, F. spiralis* and *F. serratus*. All other seaweed extracts tested had a level of phenolic compounds less than 25 GAE mg^{-1}. Brown seaweeds accumulate a variety of phloroglucinol-based polyphenols (phlorotannins), formed from the polymerization of phloroglucinol monomer units resulting in compounds with different molecular weights. Phlorotannins are stored in special vesicles and are presumed to be defense compounds involved in protection against stress conditions and herbivores [51]. Phlorotannins are reported to act as inhibitory compounds for anaerobic digestion as they inhibit enzyme activity of various microbes [5]. Other polyphenolic compounds previously quantified in brown seaweeds include caffeic acid, chlorogenic acid, coumaric acid and catechins. Phlorotannins are unique to marine algae and are found at highest levels (2-10%) in brown algal species. [52]. Depending on the structure of the polyphenols contained in the extract, it is likely that portions of the phenolic content are the causative agent for the inhibition seen at a concentration of 2.5 mg/ml of *F. serratus* extract.

Fermentation in the gut can be considered in terms of a complex assortment of bacterial metabolic pathways, with each bacterium trying to garner the energy required for survival from the finite supply of carbon. In this system, fermentation end-products from one bacterial species can be utilised by another which lack the ability to ferment the original source. Further investigation of the bifidogenic or prebiotic potential of seaweeds would also require substantial refinement of the extract to be investigated. The extracts used in this study were obtained through a solid liquid extraction process with cold water being used as the solvent. It was shown that several of these extracts, following the extraction process, contained appreciable amounts of glucose as well as large concentrations of phenolic compounds. Simple monosaccharides are universally used by bacteria as a source of energy for growth. One of the key principles of the prebiotic concept is that of selective stimulation of beneficial bacteria. In experimental models, the presence of monosaccharides would serve to facilitate the growth of both beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* but also potentially harmful members of the microbiota and pathogens. Also, dietary phenolic compounds,

at high concentrations, are known to exhibit anti-microbial activity and could explain the inhibitory effect observed at the higher concentrations of *F.* stratus extract. As such, both simple sugars and phenolic compounds are to be regarded as undesirable in terms of the preparation of an extract to be used in bifidogenic/prebiotic trials. Any extraction process to be used in future prebiotic investigations of seaweeds should be designed in such a manner as to address these issues. Several techniques could be added to the extraction process to generate specific extracts for prebiotic investigation. To further increase the yield of total carbohydrates and to release bound polysaccharides from their cell wall matrices, a more vigorous and targeted extraction mechanism may be required for the preparation of extracts in the future. An example of this would be the use of a dilute hot acid extraction instead of using cold water as the extraction solvent. The introduction of an ethanol precipitation step immediately following the primary extraction would cause separation of seaweed monosaccharides, polysaccharides and dietary fibre from the non-carbohydrate contents of the seaweed. Further, size exclusion dialysis could also be employed to remove the seaweeds monosaccharide content, such as glucose, from the final extract. Based on the results obtained herein, the brown seaweed *F. serratus* proved to be the best candidate seaweed for further prebiotic investigation. Further use of this seaweed in prebiotic potential investigations is recommended. The adoption of a much-modified extraction methodology is also advised to give further prebiotic studies the best chance of success.

2.6 Conclusion.

In conclusion, the results presented here indicate that different bifidobacterial strains can successfully utilize the carbohydrate content of brown, red and green seaweed as their sole carbon source, as determined by an increase in optical density (OD600nm). All tested seaweeds (*A. esculenta, A. nodosum, C. crispus, C. fragile, F. serratus, G. gracilis, H. elongata, L. digitata, L. hyperborea, P. canaliculata,* and *U. intestinalis*) had a significant effect on the growth of one or more of the *Bifidobacterium* strain used in this study. The greatest effect was found with the brown seaweeds, *F. serratus* and *S. latisima*, with *F. serratus* being chosen for further evaluation. A dose response analysis was carried out with the *F. serratus* extract. Significant bifidogenic activity was detected with the *F. serratus* extract at all concentrations for *B. breve, B. lactis* and *B. longum.* The greatest bifidobacteria stimulatory effect was observed with the *F. serratus* extract at a concentration of 1.25 mg/ml. It was also shown that this concentration the *F. serratus* extract had a significantly greater stimulatory effect *B. longum* than the FOS prebiotic control. An unexpected inhibitory effect was observed at a concentration of 2.5 mg/ml for the *F. serratus* extract, most probably because of the presence of large quantities of phenolic compounds, which are known to possess antimicrobial activity. While the results presented here were positive in terms of bifidogenic potential, the effect was strain dependent. Further, information is lacking with regards to the effect of the extracts on mixed bacterial cultures. It is recommended that further investigation of the prebiotic potential of brown seaweeds be undertaken, with an emphasis on *F. serratus*, using an *ex vivo* faecal fermentation system and a revised extraction method.

2.7 References.

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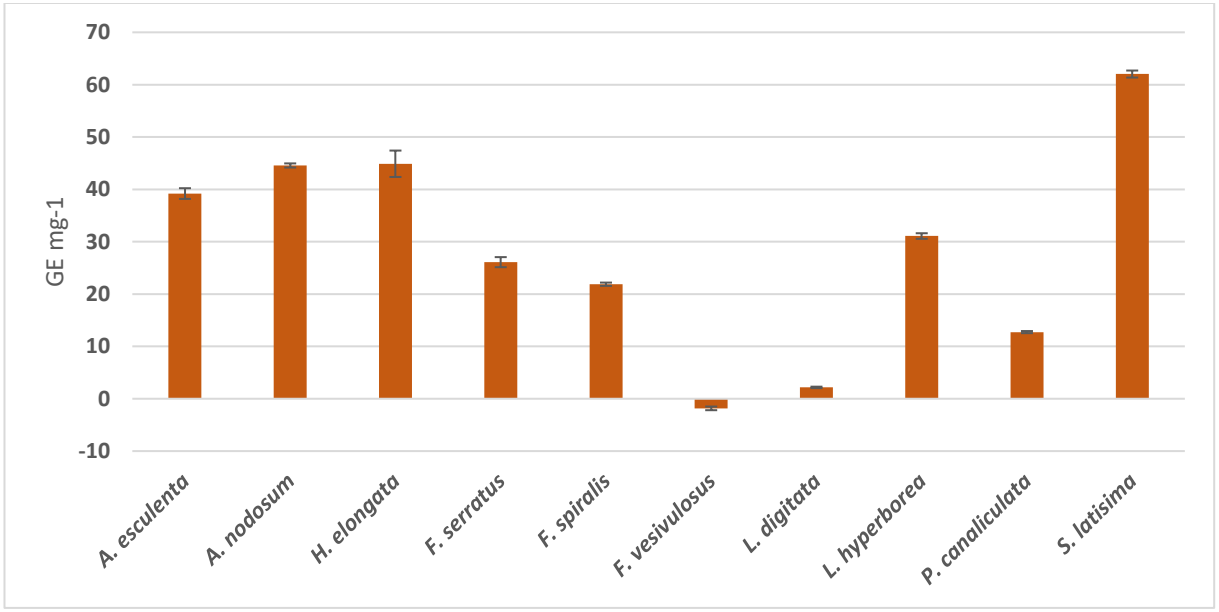
Figure 2.1 Total carbohydrate analysis using a resorcinol-sulphuric acid microplate method with modifications for (a) brown seaweed species and (b) red and green seaweed species. Data represent the mean (± SE).

(a)



(b)

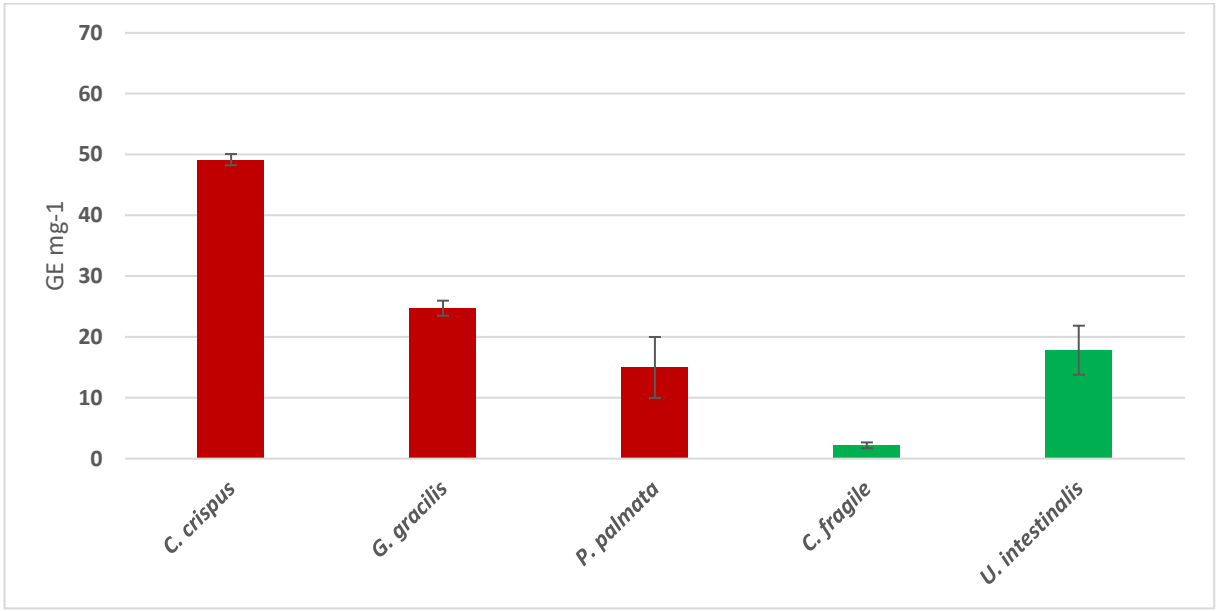


Figure 2.2 The concentration of the monosaccharides glucose and galactose was determined for the ten brown seaweed extracts using a HPLC system equipped with a reactive index detector.

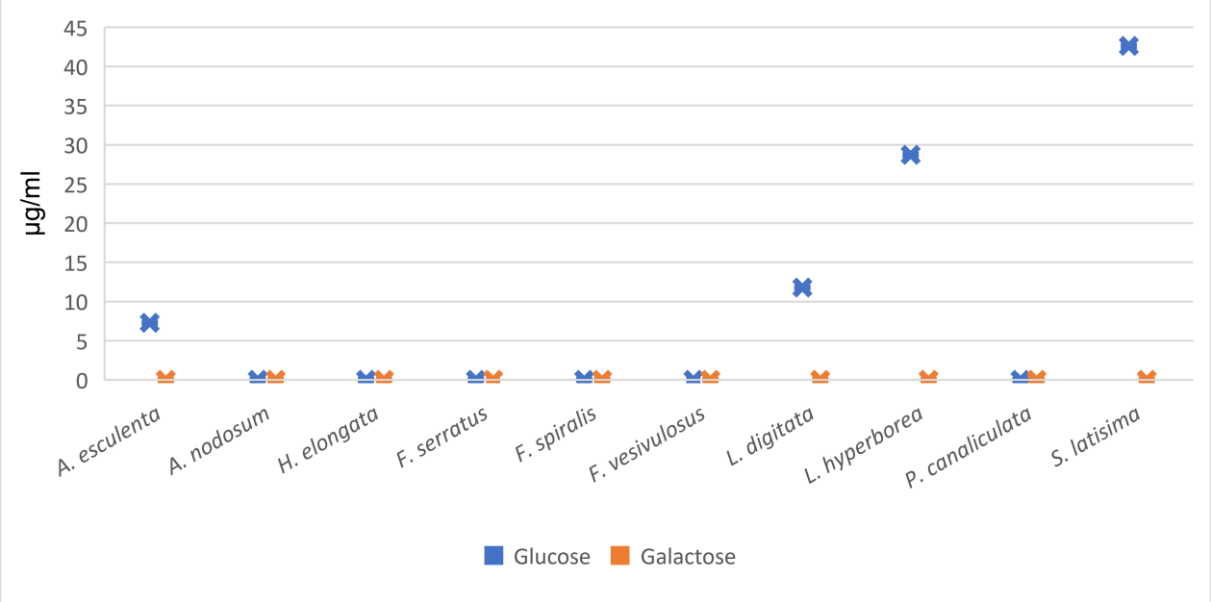
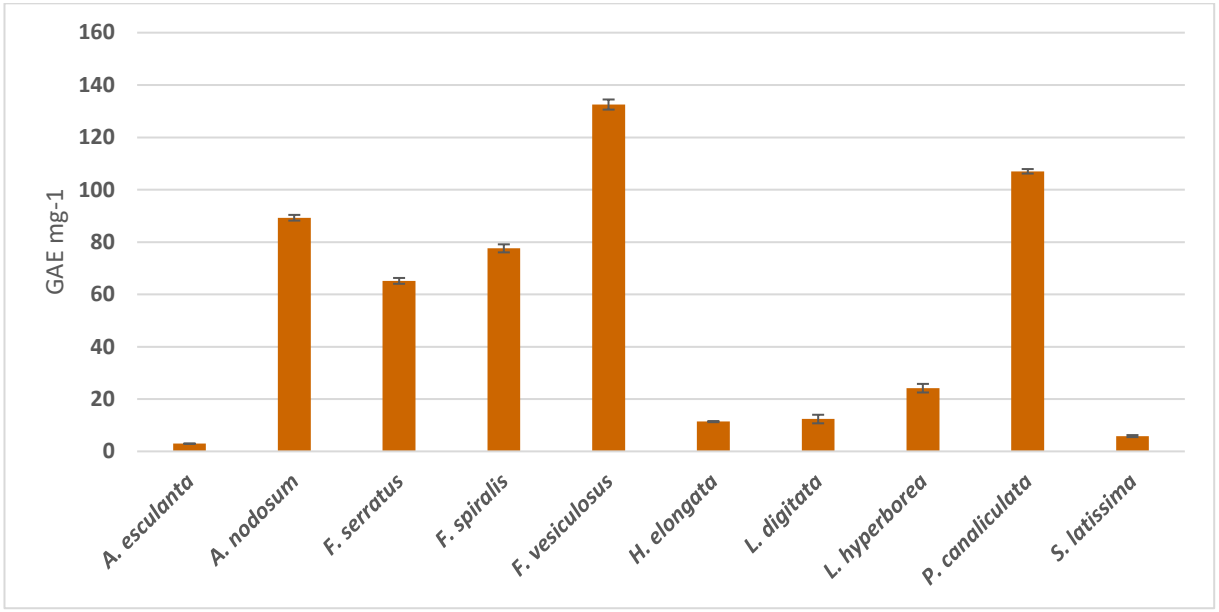


Figure 2.3 Total Phenolic Content (TPC) of (a) the brown seaweed species and (b) the red and green seaweed species used in this study. Data represent the mean (± SE).

(a)



(b)

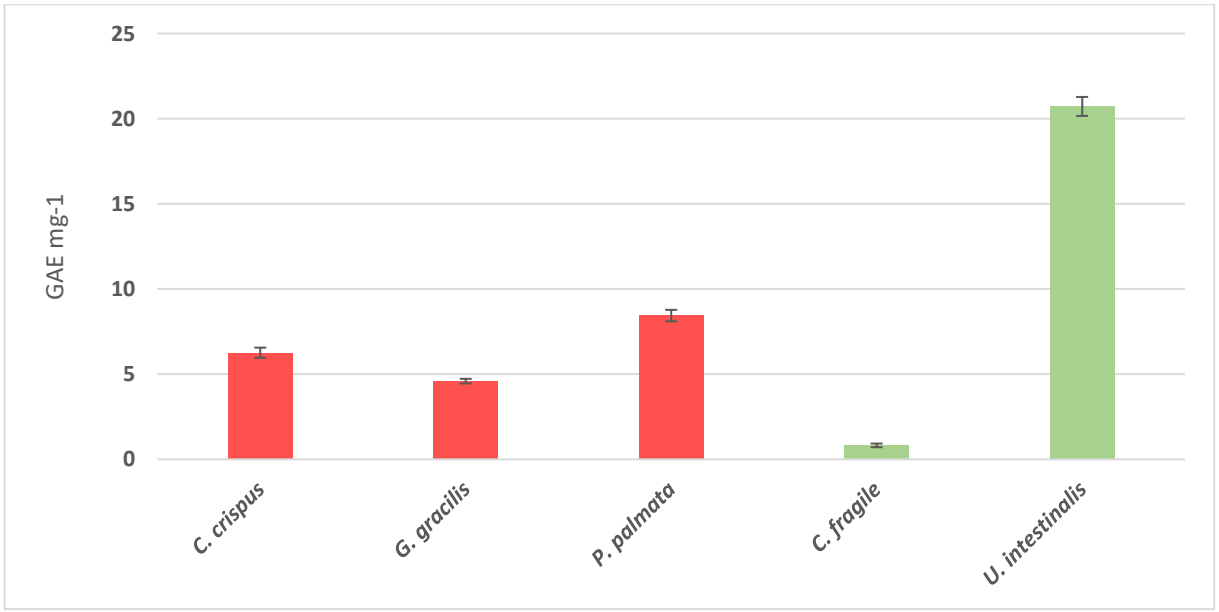


Figure 2.4 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium breve* APC 325 NCBF 8807. Growth was measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

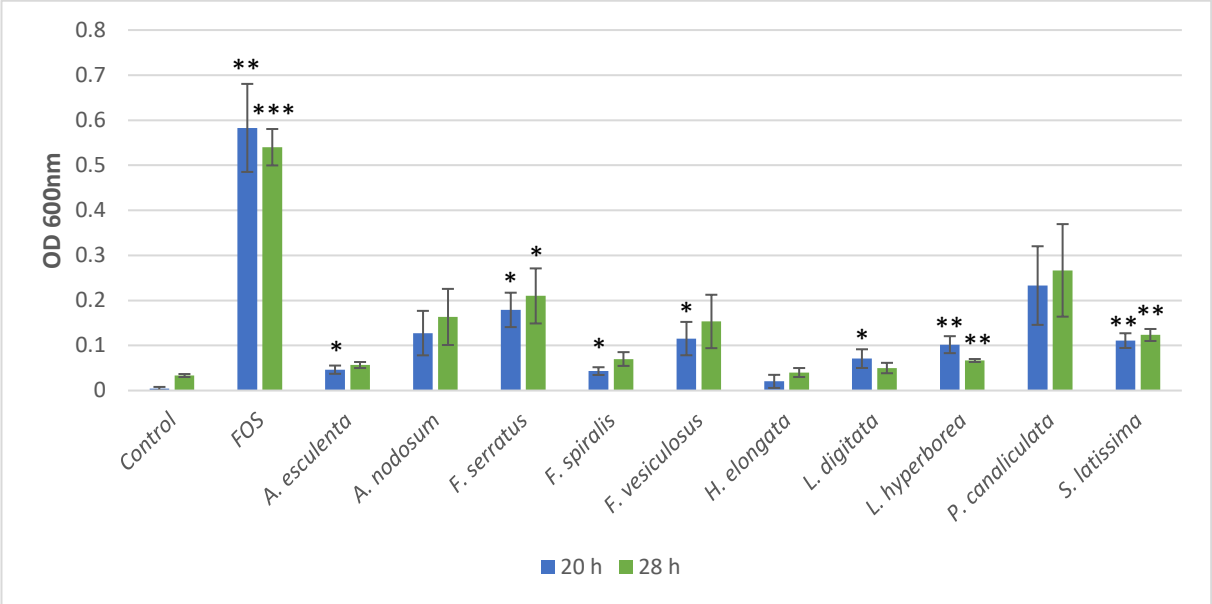


Table 2.1 Percentage change in OD600nm readings for the ten Irish brown seaweed extracts in comparison with the cellulose control for *Bifidobacterium breve* APC 325 NCBF 8807.

% Change	20 h	28 h
FOS	10907.8 ± 3636.5	1590.2 ± 295.6
A. esculenta	855 ± 408.4	63.5 ± 26.6
A. nodosum	2957.4 ± 1555.3	464.4 ± 252.4
F. serratus	3498.5 ± 1175.6	555.3 ± 194.4
F. spiralis	894.7 ± 438.9	124.5 ± 68.3
F. vesiculosus	1795.3 ± 253.6	342.8 ± 159
H. elongata	405.8 ± 467.6	37.3 ± 59.5
L. digitata	1420 ± 833.1	51.8 ± 33.3
L. hyperborea	2115.6 ± 1018.7	114.7 ± 46.2
P. canaliculata	4800.2 ± 2054.7	755.9 ± 323.5
S. latissima	2128.1 ± 865.1	293.6 ± 96.8

Figure 2.5 The effect of cold-water extracts from Irish red and green seaweeds on the growth of *Bifidobacterium breve* APC 325 NCBF 8807. Growth was measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

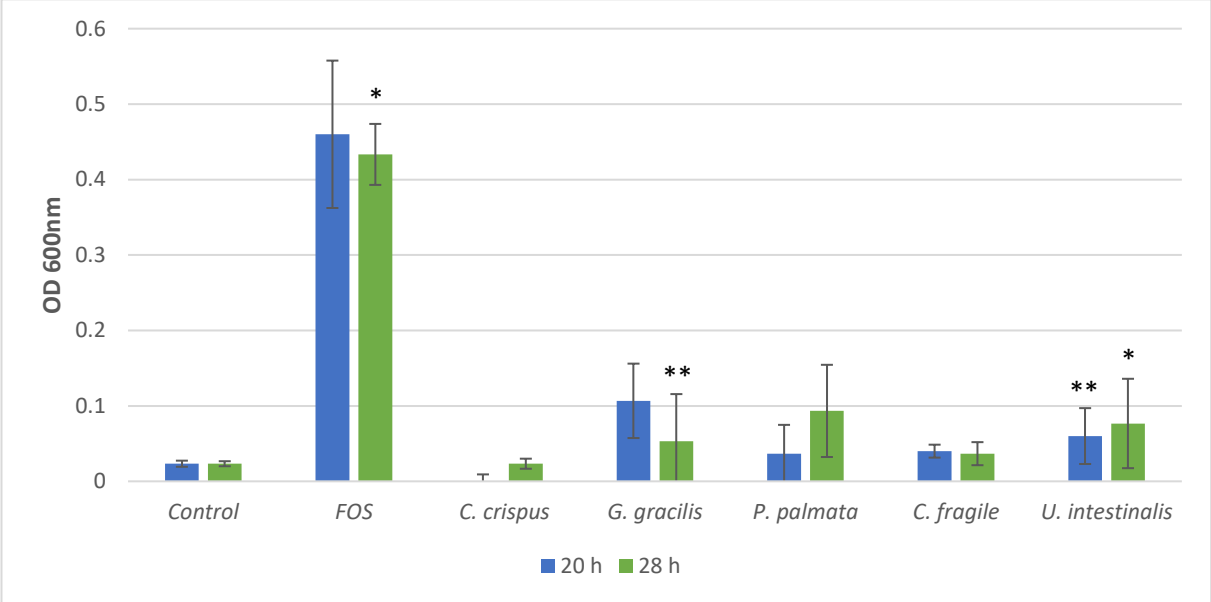


Table 2.2 Percentage change in OD600nm readings for the Irish red and green seaweed extracts in comparison with the cellulose control for *Bifidobacterium breve* APC 325 NCBF 8807.

% Change	20 h	28 h
FOS	1762.1 ± 626.6	1828.8 ± 725.3
C. crispus	-88.8 ± 69.4	-13.9 ± 84.8
C. fragile	68.7 ± 33.3	118.1 ± 72.4
G. gracilis	337.1 ± 109.7	282.8 ± 55.9
P. palmata	42.5 ± 34.5	43 ± 18.5
U. intestinalis	148.8 ± 39	203.9 ± 35.3

Figure 2.6 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium lactis* Bb12 APC 326. Growth was measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

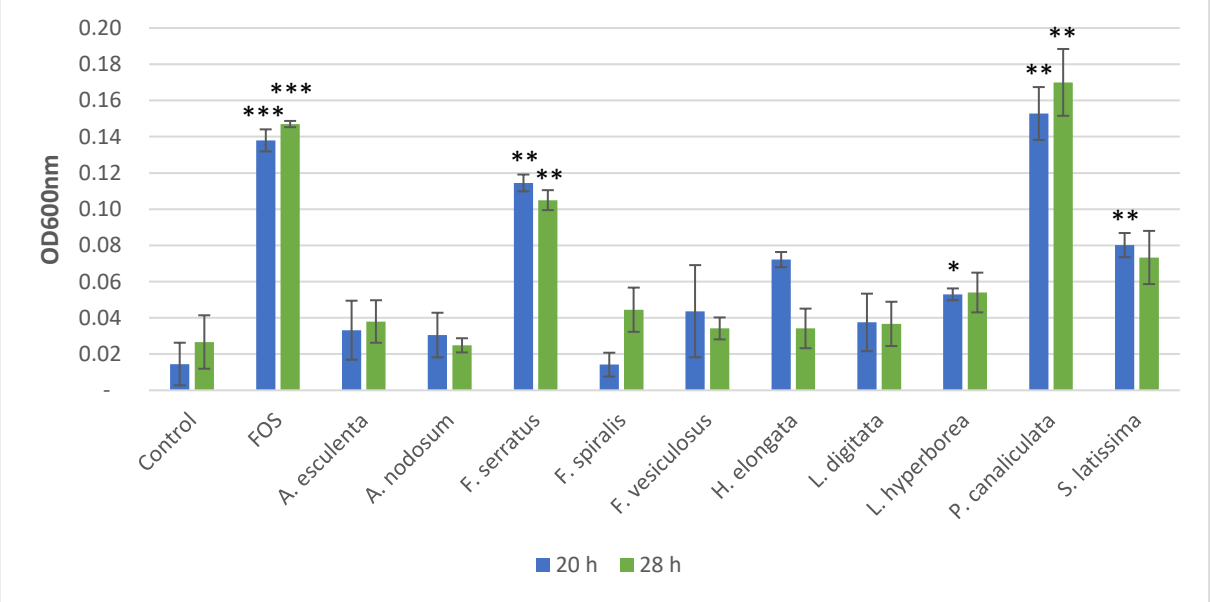


Table 2.3 Percentage change in OD600nm readings for the ten Irish brown seaweed extracts in comparison with the cellulose control for *Bifidobacterium lactis* Bb12 APC 326.

% Change	20 h	28 h
FOS	836.2 ± 406.2	2659.9 ± 2395.3
A. esculenta	181.2 ± 177.7	1001.5 ± 1036.8
A. nodosum	107.9 ± 59	534.7 ± 582.7
F. serratus	664.6 ± 307.4	1967.9 ± 1816.1
F. spiralis	23.8 ± 43.8	1155.9 ± 1172.2
F. vesiculosus	125.8 ± 55	430.2 ± 435
H. elongata	417.5 ± 241.4	689.7 ± 706
L. digitata	125.5 ± 20.7	678.1 ± 698.9
L. hyperborea	289.5 ± 180.4	881.8 ± 835.1
P. canaliculata	857.7 ± 314.8	3646 ± 3364.8
S. latissima	495.1 ± 308.1	1524.4 ± 1451.1

Figure 2.7 The effect of cold-water extracts from Irish red and green seaweeds on the growth of *Bifidobacterium lactis* Bb12 APC 326. Growth was measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

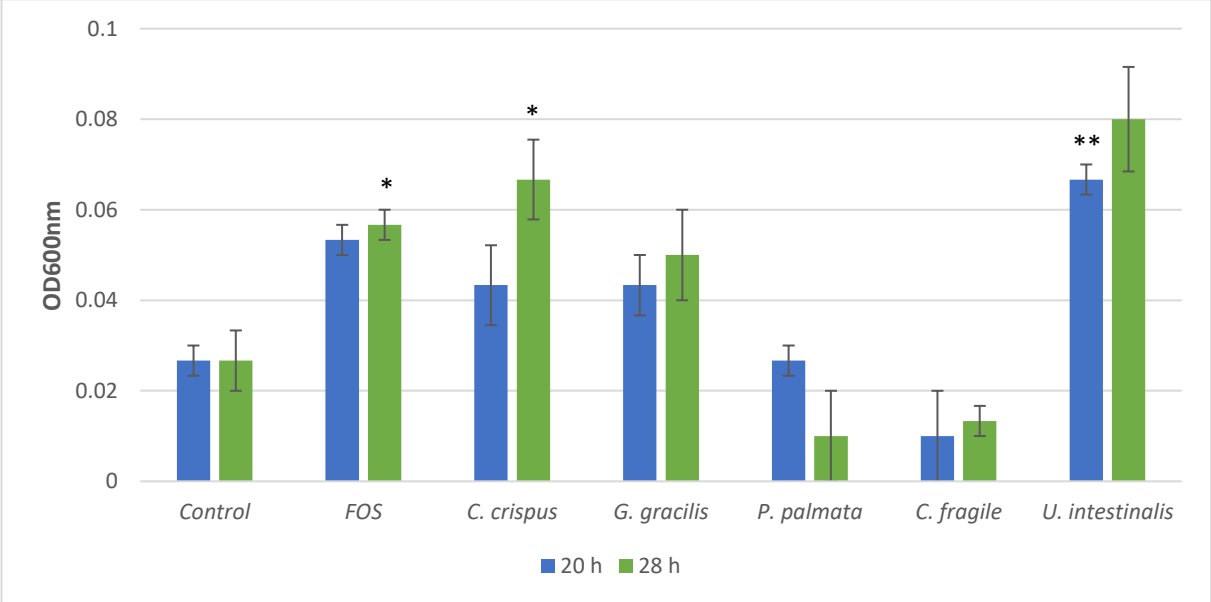


Table 2.4 Percentage change in OD600nm readings for the Irish red and green seaweed extracts in comparison with the cellulose control for *Bifidobacterium lactis* Bb12 APC 326.

% Change	20 h	28 h
FOS	118.5 ± 25.5	113.1 ± 48.7
C. crispus	83.6 ± 31.4	146.6 ± 45.6
C. fragile	-45.4 ± 42.5	-53.2 ± 18.2
G. gracilis	76 ± 46.6	89.7 ± 56.6
P. palmata	9.7 ± 34.7	-47.3 ± 32.8
U. intestinalis	173.5 ± 58.1	186.7 ± 35.6

Figure 2.8 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium longum* APC 422 DPC 6205. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

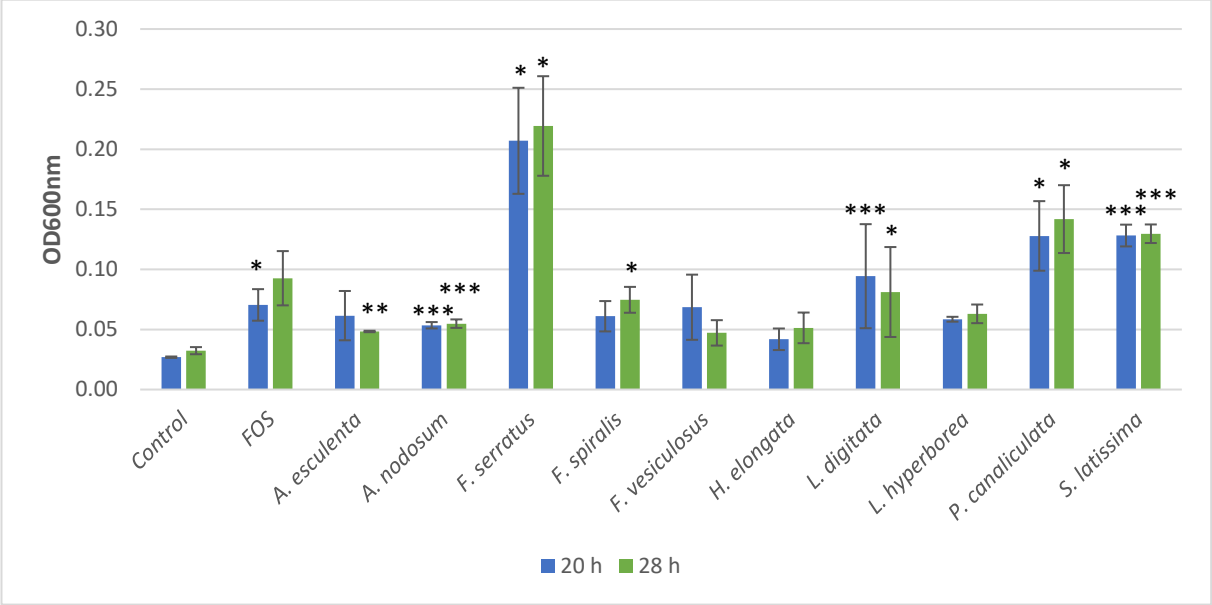


Table 2.5 Percentage change in OD600nm readings for the ten Irish brown seaweed extracts in comparison with the cellulose control for *Bifidobacterium longum* APC 422 DPC 6205.

% Change	20 h	28 h
FOS	163.3 ± 51.9	60.3 ± 11
A. esculenta	129.2 ± 77.2	33.1 ± 6.4
A. nodosum	99.1 ± 11.8	40 ± 9
F. serratus	676.5 ± 180.8	83.8 ± 4.3
F. spiralis	128.7 ± 51.9	53.9 ± 10.7
F. vesiculosus	154.6 ± 101.7	27.2 ± 10.7
H. elongata	54.4 ± 31.1	29 ± 16.4
L. digitata	256.7 ± 169.8	42.1 ± 20.5
L. hyperborea	117.4 ± 6	48.2 ± 3.3
P. canaliculata	373.1 ± 105.2	75.4 ± 4.9
S. latissima	376.6 ± 34.9	74.9 ± 2.9

Figure 2.9 The effect of ten cold-water extracts from Irish red and green seaweeds on the growth of *Bifidobacterium longum* APC 422 DPC 6205. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

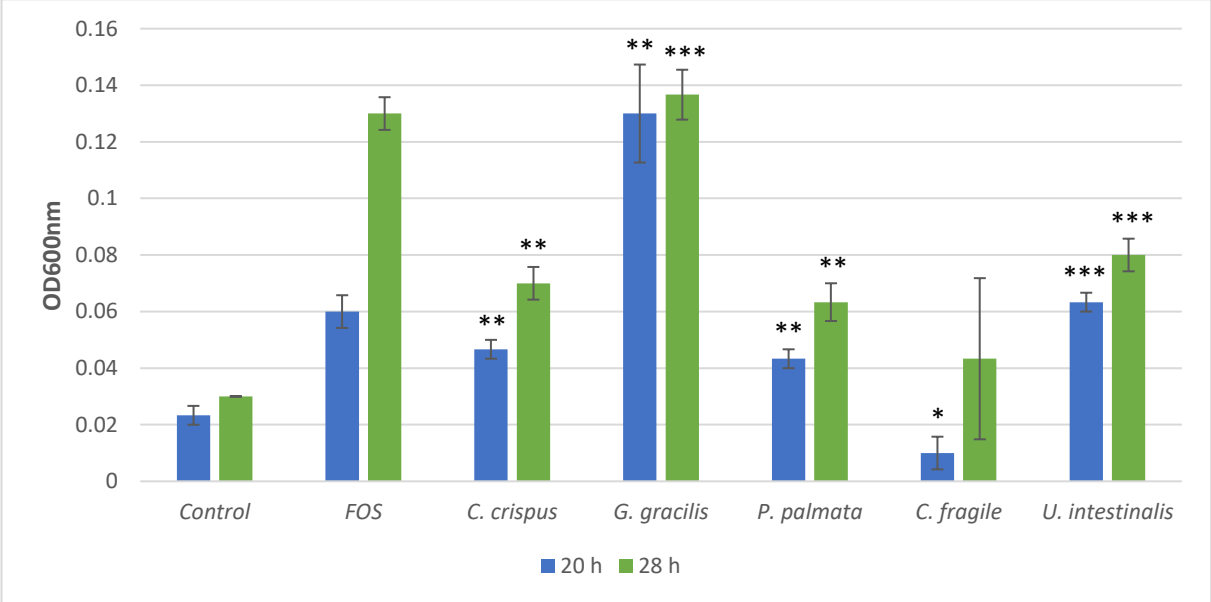


Table 2.6 Percentage change in OD600nm readings for the Irish red and green seaweed extracts in comparison with the cellulose control for *Bifidobacterium longum* APC 422 DPC 6205.

% Change	20 h	28 h
FOS	157.9 ± 31.4	361.8 ± 44.8
C. crispus	91.8 ± 16.8	2.5 ± 0.3
C. fragile	-54.9 ± 14.9	0.9 ± 1.7
G. gracilis	448.4 ± 46.1	6.2 ± 0.6
P. palmata	89.2 ± 8.4	2.1 ± 0.3
U. intestinalis	167.9 ± 20.9	3.1 ± 0.2

Figure 2.10 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium bifidum* DPC 6034. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats.

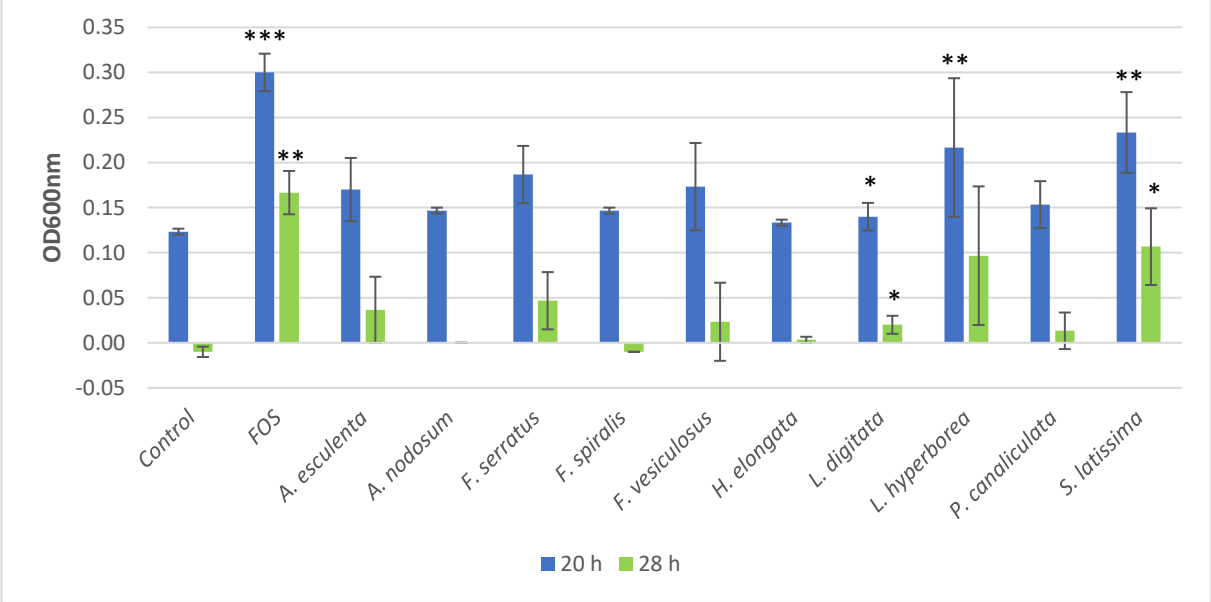


Table 2.7 Percentage change in OD600nm readings for the ten Irish brown seaweed extracts in comparison with the cellulose control for *Bifidobacterium bifidum*.

% Change	20 h	28 h
FOS	143.7 ± 25.5	2129.7 ± 771.7
A. esculenta	34.7 ± 22.8	1163.3 ± 1039.9
A. nodosum	18.8 ± 4.2	93.4 ± 11
F. serratus	48.4 ± 20.8	1165.7 ± 981.8
F. spiralis	16 ± 3.2	-54 ± 101.7
F. vesiculosus	37.4 ± 30.3	1117.3 ± 1127.3
H. elongata	6.9 ± 1.2	212.7 ± 86.6
L. digitata	12 ± 5.7	506.2 ± 339.9
L. hyperborea	68.5 ± 52.8	2563.1 ± 2312
P. canaliculata	22.4 ± 14	544.5 ± 453
S. latissima	84.4 ± 26.4	2242.9 ± 1668.2

Figure 2.11 The effect of ten cold-water extracts from Irish red and green seaweeds on the growth of *Bifidobacterium bifidum DPC 6034*. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test against cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeats**.**

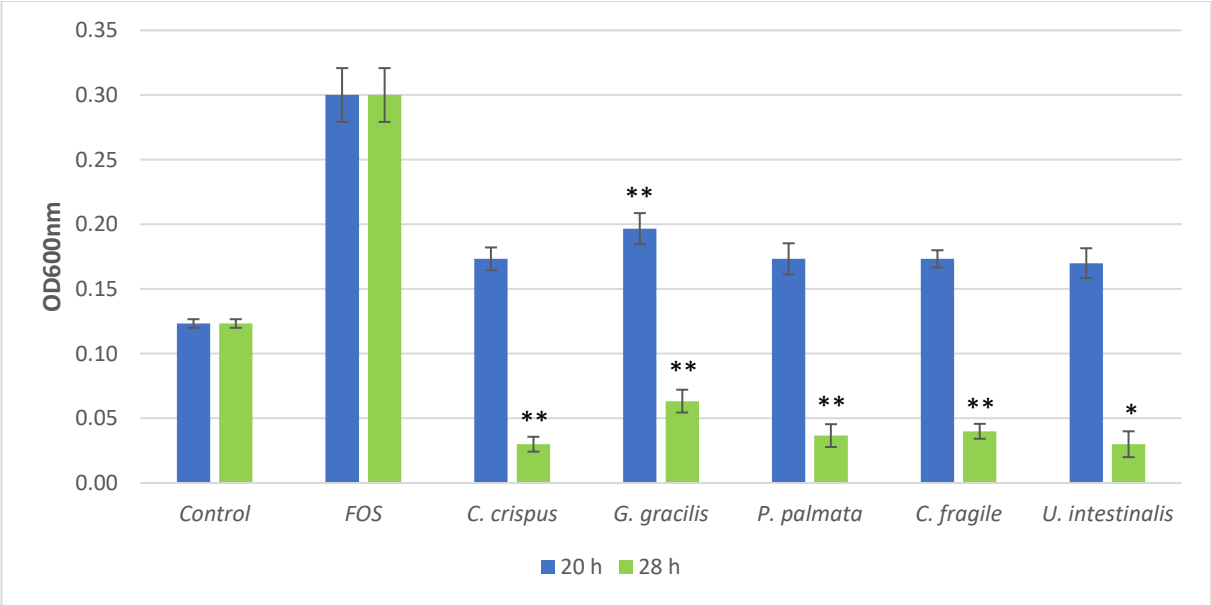


Table 2.8. Percentage change in OD600nm readings for the Irish red and green seaweed extracts in comparison with the cellulose control for *Bifidobacterium bifidum*.

% Change	20 h	28 h
FOS	143.7 ± 25.5	2129.7 ± 771.7
C. crispus	37.8 ± 1.5	599 ± 338.4
C. fragile	57.7 ± 2	1096.4 ± 647.3
G. gracilis	37.3 ± 4.7	757 ± 432.3
P. palmata	37 ± 7.1	697.5 ± 337.1
U. intestinalis	36.4 ± 8	593.6 ± 349.1

Figure 2.12 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium longum* subspecies *infantis* DPC 6036. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeat.

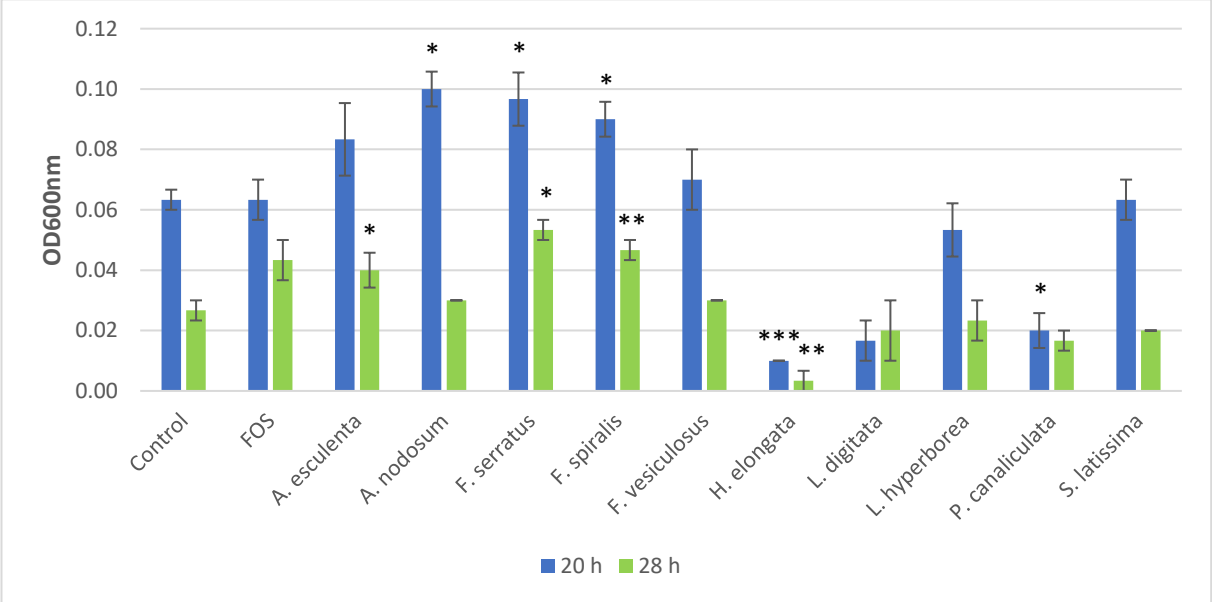


Table 2.9 Percentage change in OD600nm readings for the ten Irish brown seaweed extracts in comparison with the cellulose control for *Bifidobacterium longum* subspecies *infantis* DPC 6036.

% Change	20 h	28 h
FOS	0.9 ± 12.6	70 ± 35.1
A. esculenta	36.2 ± 24.7	50.9 ± 19.6
A. nodosum	59.1 ± 18.9	28.1 ± 19.9
F. serratus	50.7 ± 16.5	108.4 ± 25.3
F. spiralis	46.6 ± 17.8	72.8 ± 28.9
F. vesiculosus	15.7 ± 19.5	9.8 ± 16.9
H. elongata	-83.6 ± 3.3	-93.7 ± 9.2
L. digitata	-74 ± 5.4	-23.1 ± 33.6
L. hyperborea	-14.7 ± 9.7	-10.6 ± 30.7
P. canaliculata	-71.5 ± 9.1	-36.7 ± 19.9
S. latissima	4.2 ± 9.8	-21.2 ± 14.5

Figure 2.13 The effect of ten cold-water extracts from Irish brown seaweeds on the growth of *Bifidobacterium longum* subspecies *infantis* DPC 6036. Growth measured by readings of optical density (OD600nm). Data represent the mean (± SE). Significance was measured by independent t-test cellulose negative control. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeat.

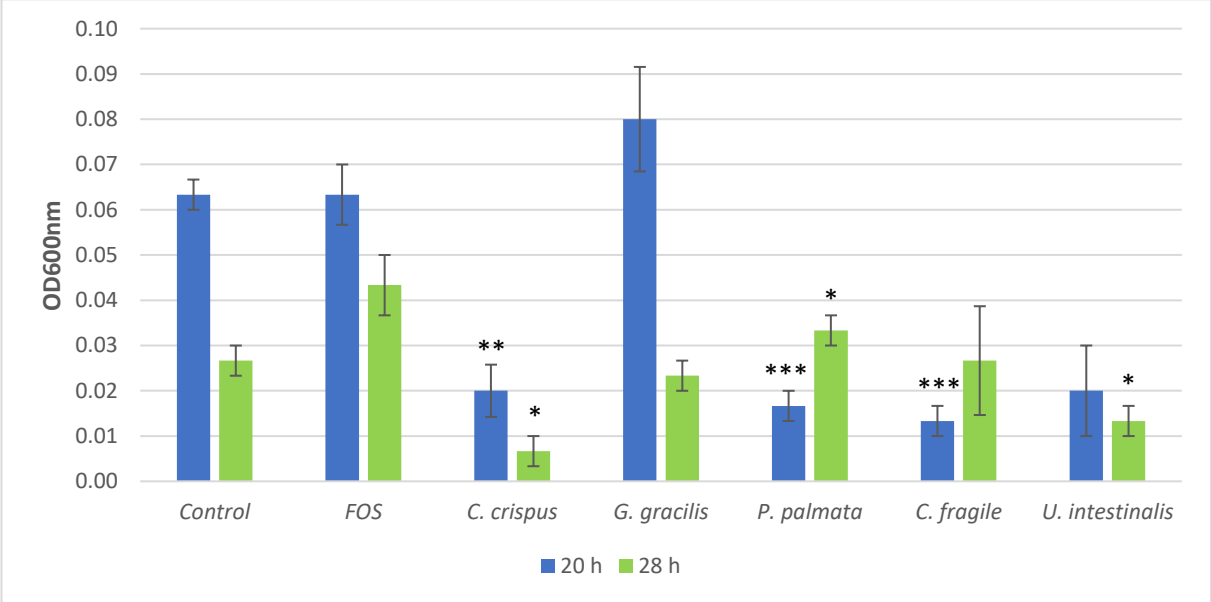


Table 2.10 Percentage change in OD600nm readings for the Irish red and green seaweed extracts in comparison with the cellulose control for *Bifidobacterium longum* subspecies *infantis* DPC 6036.

% Change	20 h	28 h
FOS	0.9 ± 12.6	70 ± 35.1
C. crispus	-70.6 ± 9.9	-70.9 ± 12.3
C. fragile	23.3 ± 23.5	-14.5 ± 7.8
G. gracilis	-76.1 ± 2.8	37.5 ± 16
P. palmata	-84.1 ± 5.2	-13.4 ± 33.4
U. intestinalis	-68.1 ± 15.4	-45 ± 4.9

Figure 2.14 Effect of different concentrations of the *Fucus serratus* extract on the growth of (a) *Bifidobacterium breve* APC 325 NCBF 8807, (b) *Bifidobacterium lactis* Bb12 APC 326 and (c) *Bifidobacterium longum* APC 422 DPC 6205. Data represent the mean (± SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test)

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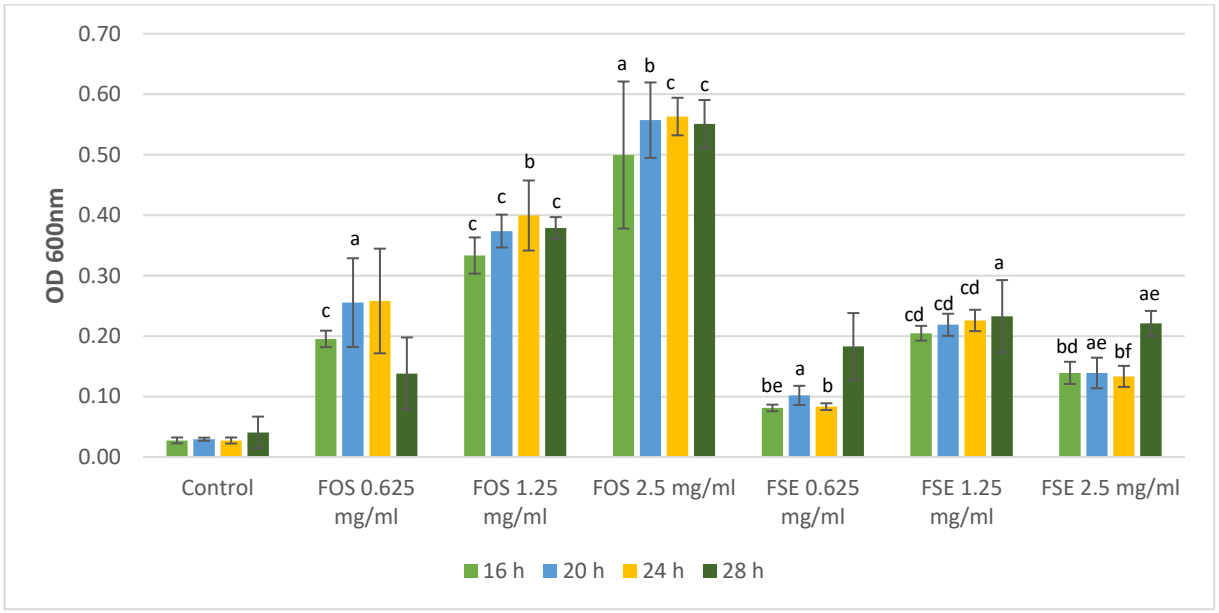
(a)



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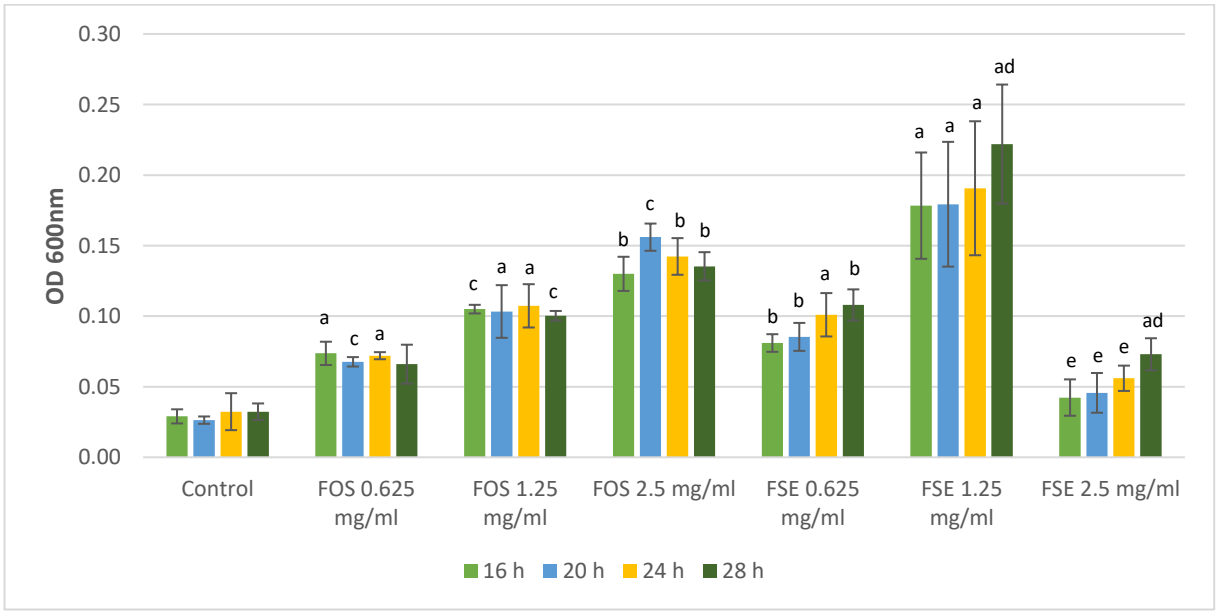

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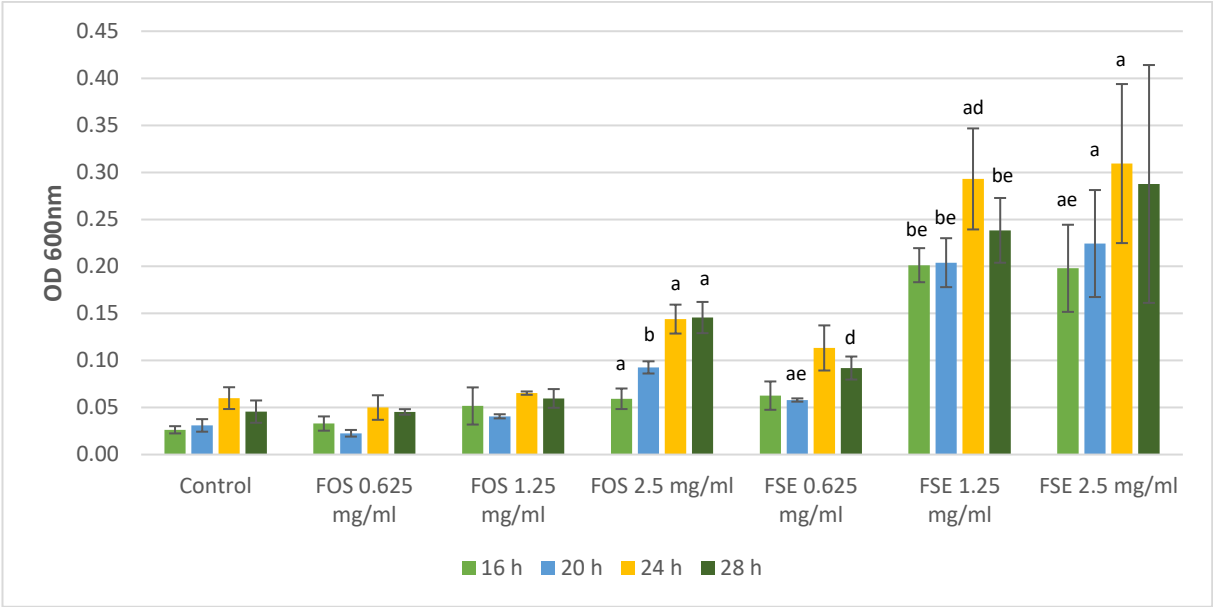
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Control
FOS 0.625 mg/ml
FOS 1.25 mg/ml
FOS 2.5 mg/ml
FSE 0.625 mg/ml
FSE 1.25 mg/ml
FSE 2.5 mg/ml
16 h
20 h
24 h
28 h

Table 2.11 Percentage change in OD_{600nm} readings with the *Fucus serratus* extract and FOS control for (a) *Bifidobacterium breve* APC 325 NCBF 8807, (b) *Bifidobacterium lactis* Bb12 APC 326 and (c) *Bifidobacterium longum* APC 422 DPC 6205. Data represent the mean (± SE).

(a) B. breve

	16 h	20 h	24 h	28 h
FOS 0.625 mg/ml	687.944 ± 222.905	807.897 ± 321.081	1075.808 ± 643.569	233.543 ± 87.906
FOS 1.25 mg/ml	1158.579 ± 133.257	1195.665 ± 186.74	1588.466 ± 627.371	1754.824 ± 1125.057
FOS 2.5 mg/ml	1667.64 ± 147.84	1783.473 ± 111.598	2117.459 ± 438.388	2327.562 ± 1225.842
FSE 0.625 mg/ml	223.782 ± 84.093	245.998 ± 49.941	240.33 ± 98.411	996.89 ± 859.376
FSE 1.25 mg/ml	708.761 ± 190.698	646.473 ± 63.466	826.696 ± 276.343	681.568 ± 235.194
FSE 2.5 mg/ml	444.172 ± 127.261	367.206 ± 58.911	427.322 ± 118.618	1056.881 ± 739.281

(b) B. lactis

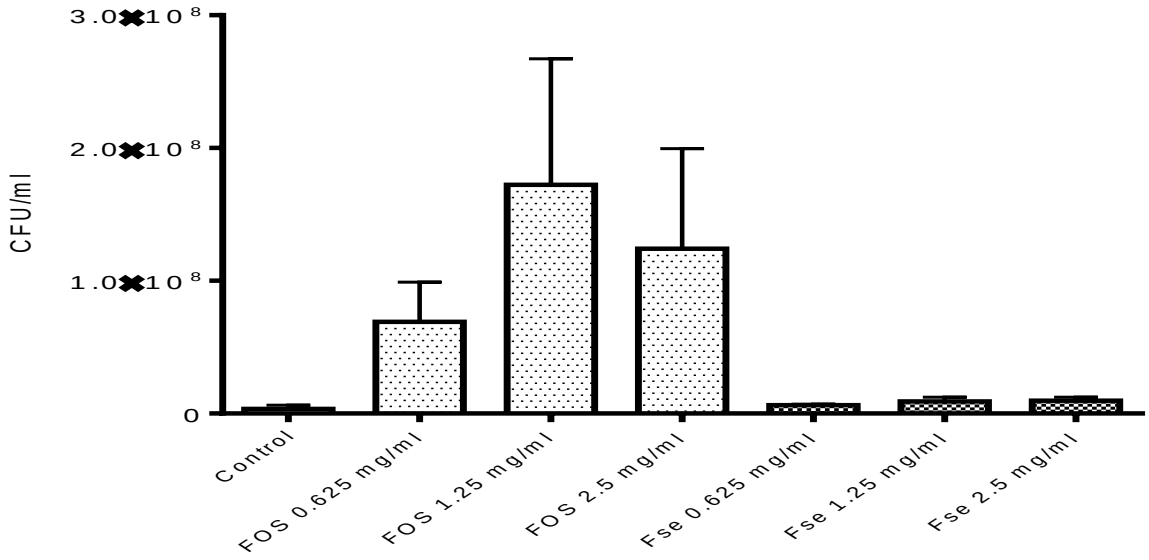
	16 h	20 h	24 h	28 h
FOS 0.625 mg/ml	176.834 ± 64.669	165.104 ± 31.407	205.125 ± 109.77	130.192 ± 73.727
FOS 1.25 mg/ml	287.35 ± 67.394	318.397 ± 117.751	398.013 ± 236.253	236.05 ± 59.31
FOS 2.5 mg/ml	384.395 ± 104.006	513.198 ± 77.337	534.004 ± 241.2	356.429 ± 91.038
FSE 0.625 mg/ml	194.24 ± 46.449	228.763 ± 30.447	300.455 ± 111.201	252.255 ± 51.693
FSE 1.25 mg/ml	530.715 ± 138.713	573.332 ± 137.096	599.186 ± 191.161	622.702 ± 166.067
FSE 2.5 mg/ml	45.375 ± 40.569	66.403 ± 42.506	112.022 ± 46.586	132.955 ± 31.693

(c) B. longum

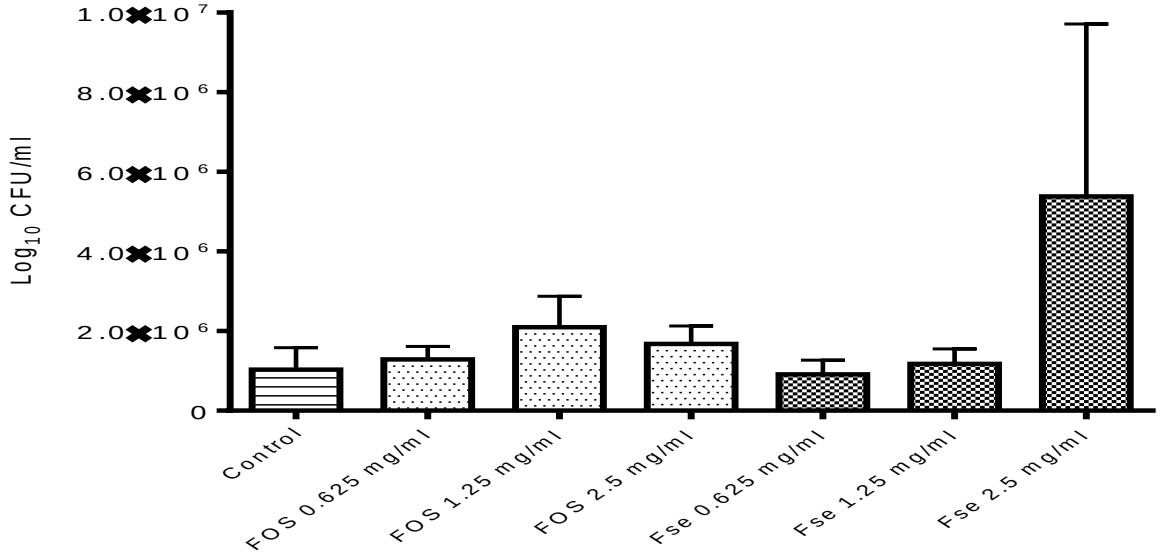
	16 h	20 h	24 h	28 h
FOS 0.625 mg/ml	37.66 ± 45.347	-15.993 ± 26.866	-3.465 ± 33.844	18.3 ± 39.453
FOS 1.25 mg/ml	84.902 ± 41.673	45.929 ± 34.826	16.812 ± 18.653	41.508 ± 24.274
FOS 2.5 mg/ml	122.653 ± 7.419	219.339 ± 43.126	156.771 ± 49.489	251.994 ± 66.314
FSE 0.625 mg/ml	156.731 ± 79.281	107.071 ± 45.314	114.414 ± 66.175	127.736 ± 52.458
FSE 1.25 mg/ml	683.493 ± 65.157	589.14 ± 75.714	418.776 ± 125.342	475.115 ± 119.314
FSE 2.5 mg/ml	642.879 ± 92.132	622.871 ± 58.176	415.272 ± 123.17	515.842 ± 152.649

Figure 2.15 Dose-response study plate counts of (a) *Bifidobacterium breve* APC 325 NCBF 8807, (b) *Bifidobacterium lactis* Bb12 APC 326 and (c) *Bifidobacterium longum* APC 422 DPC 6205. * = P<0.05, ** = P<0.005 and *** P<0.0005 vs negative control. N= 3 biological repeat**.**

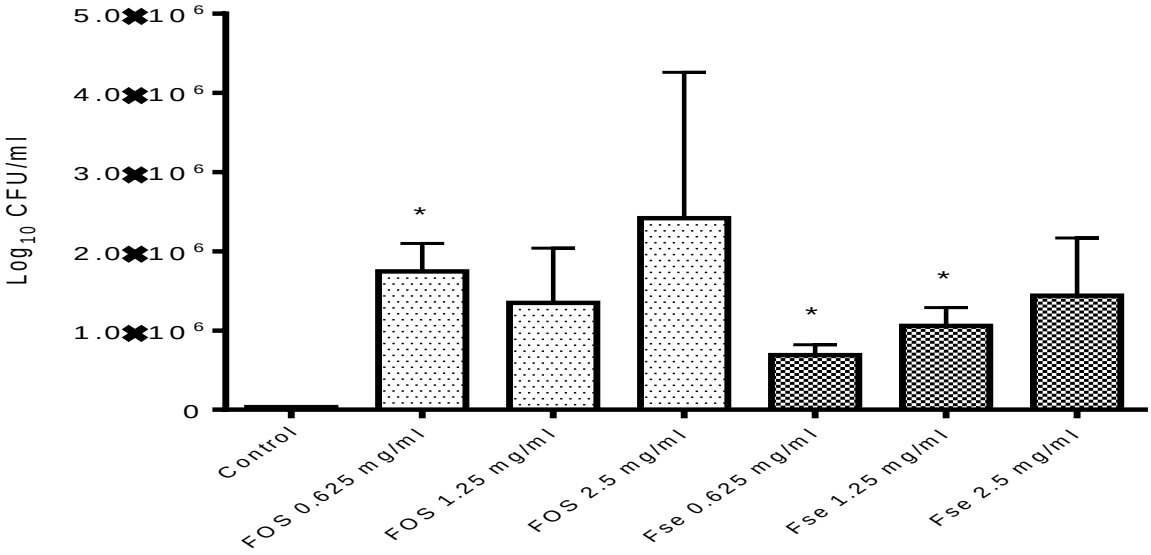
(a)



(b)



(c)


5.0✖10⁶
4.0✖10⁶
3.0✖10⁶
2.0✖10⁶
1.0✖10⁶
0
Log10 CFU/ml
*
*
*
Control
FOS 0.625 mg/ml
FOS 1.25 mg/ml
FOS 2.5 mg/ml
Fse 0.625 mg/ml
Fse 1.25 mg/ml
Fse 2.5 mg/ml

Supplementary Table 2.1 Summary of the different brown, red and green seaweed extracts used in this study. Adapted from http://www.seaweed.ie/descriptions/index.html

Group	Seaweed name	Extraction method	Other name(s)
Phaeophyta	*Alaria esculenta*	Solid-liquid extraction	Dabberlocks, Winged Kelp, Murlins
Phaeophyta	*Ascophyllum nodosum*	Solid-liquid extraction	Asco, Sea Whistle, Bladderwrack
Phaeophyta	*Fucus serratus*	Solid-liquid extraction	Serrated Wrack
Phaeophyta	*Fucus spiralis*	Solid-liquid extraction	Spiralled Wrack
Phaeophyta	*Fucus vesiculosus*	Solid-liquid extraction	Bladderwrack
Phaeophyta	*Himanthalia elongata*	Solid-liquid extraction	Thongweed, Buttonweed, Sea Spaghetti
Phaeophyta	*Laminaria digitata*	Solid-liquid extraction	Kelp
Phaeophyta	*Laminaria hyperborea*	Solid-liquid extraction	Kelp, May Weed
Phaeophyta	*Pelvetia canaliculata*	Solid-liquid extraction	Chanelled wrack
Phaeophyta	*Saccharina latissima*	Solid-liquid extraction	Sugar Kelp (formally Laminaria saccharina)
Rhodophyta	*Chondrus crispus*	Solid-liquid extraction	Irish Moss, Carragheen, Carrageen, Carrageen Moss
Rhodophyta	*Gracilaria gracilis*	Solid-liquid extraction	n/a
Rhodophyta	*Palmaria palmata*	Solid-liquid extraction	Dulse, Dillisk
Chlorophyta	*Codium fragile*	Solid-liquid extraction	n/a
Chlorophyta	*Ulva intestinalis*	Solid-liquid extraction	n/a

Supplementary Figure 2.1 Location of the four different collection points along Galway bay. Clockwise from top, (a) Spiddal, Co. Galway, (b) Mweenish Island, Co. Galway, (c) New Quay, Co Clare and (d) Finnavara, Co. Clare [1]

Chapter 3

Evaluation of the prebiotic potential of a polysaccharide-rich extract from the brown seaweed *Fucus serratus* using an *ex vivo* faecal fermentation model.

3.1 Abstract.

Several seaweed polysaccharides (fucans and laminarins etc.) are resistant to degradation by human digestive enzymes. They are recognised as dietary fibres and a possible new source of prebiotic compounds. As such, these polysaccharides pass through the GIT intact until they reach the colon, where they act as a source of fermentable substrates for components of the gut microbiota. This stimulates the production of the short-chain fatty acids (SCFAs) such as acetate, butyrate and propionate. SCFA production is a major indicator of prebiotic stimulation in the colon. Here a polysaccharide rich hot-acid extract prepared from the Irish seaweed *Fucus serratus* was investigated for prebiotic potential using *ex vivo* faecal batch fermentations. A 1.5-fold increase in the production of total SCFAs was observed, particularly in the production of propionate (2.3-fold increase) and acetate (1.4-fold increase). There was also an associated significant increase ($p<0.05$) in the proportion of propionate production, rising from 15% in the control to 24 %. There was no significant change in levels of butyrate production. High throughput DNA sequencing analysis revealed that the *F. serratus* extract had no notable effect on the abundance of members of the genera *Bifidobacterium* and *Lactobacillus*. However, there were notable increases in several propionate producing members of the microbiota such as the genus *Parabacteroides,* the family *Veillonellaceae* and the family *Erysipelotrichaceae, which is* peripherally related to the butyrate-producing superfamily *Lachnospiraceae*. These results indicate that a crude polysaccharide extract from the seaweed *F. serratus* can significantly modulate the activity of the gut microbiota, and alter the SCFA production profile by stimulating propionate producing members of the microbiota.

3.2 Introduction.

Seaweeds are a ubiquitous sight along the coastlines and maritime regions of the world. They are plant-like multi-cellular organisms that generally live attached to rocks or other hard substrata in marine environments [1]. It is unsurprising that our ancestors made extensive use of seaweeds as they were both easily accessible and in plentiful supply. Traditionally, they are classified into three main groupings based primarily on the colour of their thallus or frond. These groupings are the phaeophyta (brown seaweeds), the rhodophyta (red seaweeds) and the chlorophyta (green seaweeds) [2]. Mankind's association with seaweeds stretches back many centuries and covers a wide range of activities that include their usage in cooking, herbal medicines as well as agriculture. Edible varieties of seaweed can be highly nutritious as they contain large amounts of vitamins and minerals, proteins, dietary fibre and essential fatty acids [3]. Seaweeds have formed an integral part of the cooking process in countries of the Far East, such as Japan, China and the Republic of Korea [4] where they are often used as an ingredient in soups and salads, as well as being used as a condiment [5]. Seaweed, as a staple ingredient in both the traditional and modern Japanese diet, can constitute between 10 and 25% of total food intake for some individuals, with an average of over 1.6 kg of dry seaweed being consumed per person per year [6, 7]. The seaweeds mainly used for human nutrition are of the brown (Phaeophyta) and the green (Chlorophyta) species [8]. The exploitation of seaweeds in Europe and North America has largely been confined to the manufacturing industry [9] where their polysaccharides are used as thickening and gelling agents in foods and for industrial applications [10]. Despite their abundance, seaweeds are an underutilized natural resource from a commercial and nutrition perspective [11]. Although the production of seaweed has been increasing in recent years, use on an industrial scale has been largely confined to a limited number of commercially valuable species [12], such as *Laminaria japonica*, *Undaria pinnitafida*, *Ascophyllum nodosum* and *Hizaki fusiformis* [13].

As well as being a nutrient rich food source, seaweeds produce a multitude of biologically-active secondary compounds that are associated with many health related attributes such as anti-oxidant, anti-inflammatory, anti-cancer, anti-diabetic, and anti-viral activities [14, 15]. Seaweeds are also an excellent source of dietary fibres (DFs),

especially soluble fibres and polysaccharides. Most seaweed-derived polysaccharides are non-digestible by the human digestive system. They contain complex glycosidic linkages that the natural human reservoir of hydrolytic degrading enzymes cannot break down. Seaweed DF differs in composition, chemical structure, physiochemical properties and biological effects from terrestrial plant DF [4]. The consumption of general DF is considered important in preventing conditions such as constipation, colon cancer, cardiovascular diseases, and obesity [11] and has been shown to positively influence satiety and glucose uptake from food, following meals [9]. DF from seaweeds has been shown to have cholesterol lowering and hypotensive effects [4]. In many cases, the fibre content found in seaweeds is higher than in most terrestrial fruits and vegetables with total dietary fibre ranging from 33-50 g/100g dry weight. Further, the main components of brown seaweeds differ from that of terrestrial biomass (cellulose, hemi-cellulose and lignin) with their main structural components being comprised of alginic acid, mannitol, laminarin and fucoidan.

The main soluble polysaccharides in brown seaweeds are the fucoidans, laminarins and alginates. Fucoidans are a unique class of sulphated fucans whose composition varies with the species of seaweed [16, 17]. They have an α (1-3) backbone or repeating disaccharide units of α (1-3) and α (1-4) linked fucose residues with branches at the C2 positions. Besides fucose, fucoidans may also contain the monosaccharides, galactose, mannose, xylose, and rhamnose as well as uronic acid. Depending on the species of seaweed, and to a lesser degree the season, fucoidans may constitute 25-30% of the seaweed's dry weight [18]. Sulphated polysaccharides can interact with many types of matrix and cellular proteins owing to their chemical structure, which is rich in polyanions [19]. Laminarins are composed of β-glucans and are the principle storage polysaccharides of brown seaweeds. They are short polymers of about 20-25 glucose residues linked by β (1-3) bonds and some β (1-6) bonds [20]. Alginates are the salts of alginic acid and are the main structural polysaccharide in brown seaweeds. They are composed of mannuronic and guluronic acid [16]. Soluble polysaccharides from seaweed may also serve as a new source of novel prebiotics [21] as they escape digestion in the small intestine but undergo fermentation by the commensal microbiota in the large intestine and especially the colon [22].

The term prebiotic was originally proposed in the mid 1990's by Glenn Gibson and Marcel Roberfroid, when it was demonstrated that the fibrous oligosaccharide inulin reaches the colon intact and selectively stimulates the growth of bifidobacteria [23]. The most recent definition of a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit [24]. The three main classes of carbohydrates typically classified as prebiotics are inulin-type fructans, trans-galacto-oligosaccharides, and lactulose [25]. Since the initial prebiotic publication, researches have robustly demonstrated a 'prebiotic effect' resulting from the consumption of certain fibre-containing foods, and animal studies have suggested that prebiotics influence factors such as gut and immune function, glucose tolerance, and metabolic regulation, as well as reducing the prevalence of colon cancer [23]. Prebiotic action can also lead to improvements in the integrity of the gut mucosal barrier which lessens the likelihood of developing diarrhoea [26].

Beneficial microbes, such as *Bifidobacterium, Lactobacillus* and *Eubacterium,* usually ferment carbohydrates, do not produce harmful toxins and may cause a range of benefits for the host including enhancement of the immune system and competitive inhibition of pathogens [27]. *Bifidobacterium* are a major group of beneficial bacteria in the microbiota of healthy individuals and constitute a major target for prebiotics [28]. They improve the colonic environment by suppressing pathogens and the production of carcinogenic materials [29], while also being immunomodulatory. The bifidobacterial genomes reflect their adaptation to the human GIT environment in some cases by encoding for a variety of carbohydrate-modifying enzymes, such as glycosyl hydrolases, sugar ABC transporters, and PEP-PTS (phosphoenolpyruvate – phosphotransferase system) components. These are required for the metabolism of plant and host-derived carbohydrates. Members of the genus can utilize the complex carbohydrates that are otherwise non-digestible and reach the colon unabsorbed by the body. There the complex carbohydrates are degraded to low molecular weight oligosaccharides and in many cases to monosaccharides. The simple sugars are then converted by the microbiota to intermediates of the hexose fermentation pathway (fructose-6-phosphate shunt or 'bifid' shunt) and ultimately converted to short chain fatty acids (SCFAs) and other organic components [30]. Many non-digestible oligosaccharides (NDOs) present in the gut act as prebiotics where they can enhance

the growth of beneficial members of the microbiota. Two of the most studied NDOs are fructooligosaccharide (FOS) and galacto-oligosaccharide (GOS) [31].

SCFAs are organic acids which consist of between 1 and 6 carbon atoms. Any dietary nutrients that survive gastric passage and reach the large intestine intact are potential substrates for bacterial metabolism. SCFAs are mainly produced from the fermentation of DF, resistant starch and non-digestible carbohydrates (prebiotics) by the microbiota in the colon. This leads principally to the production of butyrate, propionate and acetate, with minor amounts of the branched SCFAs, isobutyrate, valerate and isovalerate also being formed. In addition, some gasses, such as methane, carbon dioxide and hydrogen are generated. SCFAs enter cells through diffusion or by monocarboxylate transporters and solute transporters. They activate cells through cell-surface G-protein-coupled (GPR) receptors [32]. Free fatty acid receptor 2 (FFAR2, also known as GPR43) and FFAR3 (GPR41) have been identified as endogenous receptors for SCFAs. FFAR2 expression mainly occurs in immune cells, but also in adipocytes, enterocytes and endocrine cells while FFAR3 displays a wide expression pattern (spleen, lymph nodes, bone marrow, adipose tissue and colon) [33]. Acetate preferentially activates FFAR2 *ex vivo*; propionate displays similar affinity for FFAR2 and FFAR3; and butyrate preferentially activates FFAR3 [34]. The types and amounts of SCFA produced in the colon can vary depending on the composition of the microbiota and the types of undigested fibre reaching the colon. *Bifidobacterium* for example, produce both acetate and lactate which can be converted to butyrate and propionate through cross-feeding by other members of the microbiota. SCFAs produced in the gut can serve as a nutrient source for the colonic epithelium and may also act upon GIT diseases such as colon cancer, irritable bowel disease (IBD) and colitis. SCFA production also helps to lower the pH of the gut environment, inhibiting the growth of some GIT pathogens [35]. Supplementation with SCFAs has also been investigated as a treatment for ulcerative colitis, diversion colitis and short bowl syndrome [36].

As the colonic microbiota play a key role on host health, a deep understanding of the fermentation dynamics of the bacterial populations is of significant importance. The investigation into the effects of prebiotics on gut health should not be just limited to selected bacterial groups, but to the greatest possible part of the microbial

ecosystem. Only by monitoring total population shifts will we improve our understanding of the mode of action of prebiotics and our ability to determine their role in promoting health [27]. The aim of this study was to assess the prebiotic potential of a polysaccharide rich extract produced from the brown seaweed *Fucus serratus* by using an *ex vivo* faecal fermentation. *F. serratus*, also known as the serrated wrack, is a prominent canopy-forming species that has traditionally been found along the shores of Ireland and the UK as well as in other eastern Atlantic regions and more recently has been found along the Canadian coast [37].

3.3 Materials and methods.

3.3.1 Materials.

All chemicals were obtained from Sigma-Aldrich, Dublin, Ireland unless otherwise stated.

3.3.2 Seaweed origin.

The seaweed material used in this study to produce the polysaccharide rich extract was from the brown seaweed species *F. serratus*. The seaweed sample was collected in the Galway bay region of the west coast of Ireland.

3.3.3 Primary hot-acid extraction of *F. serratus.*

Loose particulate matter from the collected seaweed was removed by washing with cold water. Afterwards the washed seaweed was stored at -20 °C before use. Prior to the extraction, the seaweed was removed from storage, mechanically blended to a fine powder and added to a reaction vessel before being re-suspended with deionised water (1:10 (w/v) seaweed/water solution). Hydrochloric acid (37%) was then added (8.25ml/L) to the seaweed/water solution to give a 0.1 M HCl solution. The vessel was allowed to shake at 75 RPM in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, Ireland) for 3 h at 70 °C. After this, the vessel was removed from the shaker and allowed to cool. The seaweed solution was filtered through a muslin bag with the filtrate being transferred to a clean storage vessel. The remaining seaweed residue was returned to the reaction vessel with fresh reagents and a second extraction was performed under the same reaction conditions as above. Again, the contents were allowed to cool before being subsequently filtered using a muslin bag with the filtrate being added to the previously stored filtrate. The combined filtrate was neutralised using NaOH (pH 6-8) and centrifuged at 5000 x g for 5 min to remove remaining residual insoluble particulate matter prior to being blast-frozen and freeze-dried. The freeze-dried crude extract power was stored at -20 °C prior to further refinement.

3.3.4 Ethanol precipitation of *F. serratus* extract.

Ethanol precipitation was performed on the crude seaweed mass, immediately following the primary extraction to separate the carbohydrate content from the remaining non-carbohydrate bulk. The freeze-dried crude extract powder was re-suspended in minimal deionised water and reacted with ethanol (100%) using a ratio of seaweed: ethanol of 1:5. The seaweed ethanol reaction mixture was then centrifuged for 5 min at 5000 x g. The supernatant was discarded and the precipitate pellet containing the seaweed sugars was collected. Fresh deionised water was then used to resuspend the pellet prior to being blast and freeze-dried

3.3.5 Size-exclusion dialysis and simulated digest of the *F. serratus* extract.

Simple carbohydrates and other small compounds were removed using 1 kDa cut-off dialysis tubing (Spectrum Labs, Breda, The Netherlands). The freeze-dried extract was resuspended in minimal deionised water and dialysis tubing was cut into strips of approximately 15 cm in length. Each strip of tubing was rinsed gently with deionised water to remove traces of the sodium azide storage solution before use. The strips were filled with resuspended extract and sealed using clips. The tubing was positioned gently in a washed container filled with deionised water, covered with tin foil and placed in an orbital shaker at 25 °C at 40 rpm. The water in each container was replaced with fresh deionized water every day for three days. After the third day, the dialysis tubing was opened and the contents were collected. Following dialysis, a simulated digest was performed. Briefly, α-amylase (200 U) was dissolved in filter sterilized $CaCl_2$ (1 mM, pH 7). This was added to the seaweed mixture and incubated while shaking (150 rpm) at 37 °C for 30 min. The pH was then adjusted to pH 2 using HCl. Pepsin (2.7 g) was prepared in 125 ml 0.1M HCl and added to the seaweed mixture which was then incubated under the same conditions as before for 2 h. The pH was then adjusted to pH 7.0 with NaOH. Bile (3.5 g) and pancreatin (560 mg) prepared in 125 ml 0.5M $NaHCO_3$ were added. The mixture placed in an orbital shaker (150 rpm) for a further 3 h. Subsequently, the seaweed digest underwent a second dialysis using tubing with a molecular cut-off point of 1 kDa to remove breakdown components from the simulated digest to yield the final product.

3.3.6 Preparation of the experimental controls.

The cellulose control for this study was processed in same manner as the *F. serratus* extract. The cellulose was subjected to a simulated gastric digestion followed by size exclusion dialysis with a molecular cut off point of 1 kDa, as previously outlined. Following size-exclusion dialysis, the cellulose was blast frozen and freeze-dried. The FOS control was not subjected to a simulated digestion or size exclusion dialysis before use.

3.3.7 Carbohydrate analysis.

The total concentration of carbohydrates in the Fse extract, FOS and cellulose control was determined by a resorcinol sulphuric acid method [38] with modifications. Briefly, a 6 mg/ml resorcinol stock solution, a 14 M H_2SO_4 solution and a glucose control solution of 200 µg/ml were prepared using fresh deionised water. Each sample was prepared at a concentration of 1 mg/ml. To each microtube, 200 µl of sample, 200 µl of resorcinol solution, and 1 ml of the H_2SO_4 solution were added. Each microtube was covered in tin foil and vortexed for 30 sec. The samples were then incubated at 90 °C for 30 min after which they were placed at room temperature and allowed to cool. Readings at an optical density of 430_{nm} and 480_{nm} were taken and an average value of total carbohydrates was obtained.

To evaluate the types of polysaccharides contained within the Fse extract, the extract was degraded to its component monosaccharides in 2M trifluoroacetic acid (TFA). The Fse extract was prepared at a concentration of 1 mg/ml in the TFA and was reacted at 80 °C for 90 min with constant stirring. The concentrations of glucose and galactose in the degraded Fse extract were then determined using a HPLC method with an Aminex HPX 87C fixed ion resin column. The column was set at 60 °C and the eluent (0.009N H_2SO_4) was run at 0.5 ml/min. The sugars were detected using a refractive index detector attached to the system. Each extract sample was filtered through a 0.22 µm microfilter before subjecting them to HPLC. Calibration curves for the each sugar were generated using different concentrations 10, 20, 50, and 100 ug/ml and gave a linear response [39]

3.3.8 *Ex vivo* faecal fermentation distal colon model.

The medium used for the faecal fermentations was prepared according to Fooks et al [40]. The Fooks media consisted of: tryptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), KH_2PO_4 (0.04 g/l), K_2HPO_4 (0.04 g/l), $CaCl_2.6H_20$ (0.04 g/l), $MgSO_4.7H_2O$ (0.01 g/l), sodium bicarbonate (2 g/l), Tween 80 (2 ml/l), hemin (0.05 g/l), vitamin K1 (10 µl/l), cysteine HCl (1 g/l) and bile salts (0.5 g/l). The medium (800 ml) was pH adjusted to 6.8 and autoclaved at 121 °C for 15 min. Prior to use, each vessel of the MultiFors fermentation system (Infors UK Ltd, Surrey, UK) was autoclaved at 121 °C for 15 min. On the morning of the experiment, 2 g (1% w/v) of either control or seaweed extract (Fse) were dissolved in 160 ml of Fooks medium and added aseptically to their respective fermentation vessel. The media was sparged with nitrogen gas for at least 120 min beforehand and throughout the experiment to ensure that an oxygen-free anaerobic environment was established in the system. A minimum of three freshly voided faecal samples were collected from volunteers on the morning of the fermentation. The donors were all healthy adults (age 22 to 50 y), had no history of bowl problems and had not taken antimicrobial agents in the previous six months. The samples were combined to form a composite faecal sample in order to increase the diversity of the microbial community present within. Equal amounts from each stool sample were weighted out into a sterile filter stomacher bag (Seward, VWR, Dublin, Ireland) and then adding an appropriate volume of maximum recovery diluent (Oxoid, Fisher Scientific, Dublin) containing 0.05% L-cysteine hydrochloride adjusted to pH 6.5 (which had been boiled after autoclaving and allowed to cool in the anaerobic cabinet (Whitley A85 anaerobic workstation (DW Scientific, Shipley, United Kingdom) to give a 20% composite faecal solution. The combined samples were placed in a stomacher and homogenized for 90 sec to create the composite slurry. Immediately after homogenization, 40 ml of the faecal slurry were added to the fermentation vessels at a final volume of 200 ml. Samples (1 ml aliquots) were taken at 0 h, 5 h, 10 h, 24 h, 36 h and 48 h for total SCFA analysis, pyrosequencing analysis and direct enumeration of *Bifidobacterium* and *Lactobacillus* species. Plate counts were carried out at 0 h, 5 h, 10 h, and 24 h to enumerate the main probiotic genera, *Bifidobacterium* and *Lactobacillus.* The negative control for this experiment was cellulose and the positive control was fructooligosaccharide (FOS). The faecal

fermentation was repeated three times (n = 3) with samples being taken at each time point in duplicate.

3.3.9 Analysis of short-chain fatty acid production.

Total short chain fatty acid analysis was performed using a Varian CP-3800 GC system incorporating a Flame Ionisation Detector (FID). The system was fitted with a Zebron ZB-FFAP capillary column (30m length x 0.32 mm internal diameter x 0.32µm film thickness; Phenomenex, Cheshire, UK). Helium was supplied as the carrier gas at an initial flow rate of 1.3 ml/min. The initial oven temperature was 100 °C, maintained for 30 sec, raised to 180 °C at 8 °C/min and held for 1 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min. The temperatures of the detector and the injection port were set at 250 °C and 240 °C, respectively. Samples were taken for total SCFA analysis at 0 h, 5 h, 10 h, 36 h, and 48 h in triplicate. Each sample was centrifuged immediately at 15,000 g for 15 min to remove bacteria and other solids with the supernatant being stored at -80 °C. Prior to processing, the SCFA samples were thawed on ice, centrifuged for a further 3 min at 15,000 g and filter sterilized (0.22 µm). Samples were then diluted 1:5 with deionised water and 1 mM 2-ethylbutyric acid made up in formic acid was added to each sample as an internal control. A calibration curve was created using 10 mM, 8 mM, 4 mM, 2 mM, 1 mM, and 0.5 mM concentrations of a seven SCFA standard mix. The injected sample volume was 0.5 µl. Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Additional vials, containing standards were included in each run to maintain calibration and a cleaning injection of 1.2% formic acid was used before each analysis. The SCFAs investigated in this study were acetate, propionate, butyric acid, isobutyric acid, valerate, isovalerate and hexanoate. The internal standard used was 2 - ethylbutyric acid.

3.3.10 Enumeration of *Bifidobacterium* and *Lactobacillus* populations by plate count method.

Culturable *Bifidobacterium* and *Lactobacillus* were enumerated using agar plates counts. Samples from each fermentation vessel were taken at 0 h, 5 h, 10 h, and 24 h for bacterial plate counts. The media used for bifidobacterial enumeration was

modified de Man, Rogosa and Sharpe (MRS; Difco, Becton-Dickson ltd, Dublin, Ireland) agar plates supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma-Aldrich) and 100 µg/ml mupirocin (Oxoid). Mupirocin was prepared by adding 200 mupirocin discs (200 µg/discs) to 10 ml of mMRS broth, which was then set gently shaking on an orbital shaker for 10 min and filter-sterilized (0.45 µm) before being added to 400 ml of molten modified MRS (mMRS) agar. The mMRS agar was allowed to cool to 48 °C before the addition of the mupirocin. *Lactobacillus* selective agar (LBS; Difco, Becton-Dickson ltd, Dublin, Ireland) plates with glacial acetic acid (1.32 ml/L) were prepared for *Lactobacillus* enumeration. Serial dilutions of faecal aliquots (10^{-1} to 10^{-7}) were carried out in maximum recovery diluent (MRD) (Oxoid). Plates were incubated anaerobically in a Whitley A85 anaerobic workstation (DW Scientific, Shipley, United Kingdom) at 37 °C for 3-5 days before counting.

3.3.11 Preparation of DNA for high-throughput pyrosequencing.

Total bacterial genomic DNA was extracted from 1ml of fermentation sample that was collected at time points 0 h and 24 h and using the PowerFecal DNA Isolation Kit (MO BIO, San Diego, U.S.A). Extracted DNA was stored at - 20 °C following isolation. The microbiota composition of each sample was established by amplicon sequencing of the V4 region using universal 16S rRNA primers predicted to bind to 94.6% of all 16S rRNA genes [41, 42]. A forward primer (5'-AYTGGGYDTAAAGNG) containing a distinct multiple identifier tag (MID) for each sample (Table 3.2) and a combination of 4 reverse primers, R1 (5'-TACNVGGGTATCTAATCC), R2 (5'- CTACDSRGGTMTCTAATC), R3 (5'-TACCAGAGTATCTAATTC) and R4 (5'- TACCRGGGTHTCTAAT.CC) were utilised. All the primers used in this study were synthesised by Eurofins Genomics, Ebersberg, Germany). PCRs were carried out using an Applied Biosystems 2720 Thermo cycler (Applied Biosciences, Thermofisher, Ireland). A hot start step of 95 °C for 10 min preceded all PCR runs. Each PCR reaction was performed under the following experimental conditions: heated lid 110 °C, 94 °C for 2 min followed by 36 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. This was followed by a final temperature step of 72 °C for 2 min and a holding step at 4 °C. PCRs had a final volume of 50 µl comprising 25 µl of BioMix Red (Medical Supply Company, Dublin,

Ireland), 1 ul forward primer (0.15 μM), 1 μl reverse primer (0.15 μM) (mix of 4), 5 μl template DNA, and 18 μl sterile PCR water (BioLine, UK). All PCRs were carried out in triplicate. PCR products were analysed using agarose gel electrophoresis (1.5% in 1 x TAE buffer). The DNA products were subsequently cleaned using Agencourt AMPure XP (Beckman Coulter, California, U.S.A) and quantified using the Quant-iT™ PicoGreen ® dsDNA Assay Kit (Invitrogen, Massachusetts, U.S.A). All samples were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, U.K) per 454 protocols.

3.3.12 Analysis of sequencing data.

Raw sequences were quality-trimmed using the Qiime Suite of programmes[43]; any reads not meeting the quality criteria (a minimum quality score of 25 and a sequence length <150bp for 16S amplicon reads) were discarded. OTUs were aligned using PyNAST [44] and taxonomy assigned using BLAST [45] against the SILVA SSURef [46] database release 111. Alpha (α) and β diversity indices and rarefaction curves were generated using Qiime. Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev. A phylogenetic tree was calculated using the FastTree [47] software and the resulting principal coordinate analysis was visualised within KiNG.

3.3.13 Statistical analysis.

All results are presented as mean (± SE). Independent t-tests were used to measure significance ($p<0.05$). All statistical analysis was carried out using GraphPad Prism version 5.0 for Windows. Unpaired student t-tests were carried out on SCFA data and plate counts.

3.4 Results.

In this study, a polysaccharide extract from the brown seaweed *F. serratus* was investigated for prebiotic potential using an *ex vivo* faecal fermentation model. Samples were taken over a 48-hour period for total SCFA analysis, pyrosequencing analysis, and direct enumeration of *Bifidobacterium* and *Lactobacillus* species.

3.4.1 Carbohydrate analysis of the Fse extract.

The total concentration of carbohydrates for the Fse extract, FOS and cellulose control was quantified using a resorcinol sulphuric acid method with modifications. Carbohydrate concentration is presented in terms of Glucose equivalent (GE) per milligram. Total concentration of carbohydrates in the Fse extract was determined to be 50.432 ± 1.953 GE mg^{-1}. Total concentration of carbohydrates in the cellulose control was 105.686 ± 6.332 GE mg^{-1} and in the FOS, was 272.052 ± 4.622 GE mg^{-1}. The makeup of the Fse extract was examined by treating the extract with TFA to degrade the polysaccharides to their monosaccharide building blocks. Prior to treatment with TFA, no glucose or galactose was observed in the Fse extract prior to TFA degradation. Following treatment with TFA, the degraded extract was found to have a glucose concentration of 83.95 µg/ml, and a galactose concentration of 86.07 µg/ml (Fig. 3.1).

3.4.2 Effect on culturable *Bifidobacterium* and *Lactobacillus.*

Culturable *Bifidobacterium* were selected for using MRS_{cys} agar plates supplemented with mupirocin. All values reported are minus the baseline values recorded at time point 0 h, and are ± the SEM. Bifidobacterial numbers attained their highest level at 10 h for the Fse extract ($2.04 \times 10^8 \pm 1.6 \times 10^8$), the cellulose control ($1.163 \times 10^8 \pm 9.0 \times 10^7$) and the FOS control ($4.4 \times 10^8 \pm 2.1 \times 10^8$) (Fig. 3.2). A non-significant increase in *Bifidobacterium* numbers was observed for the Fse extract and FOS at 10 h in comparison with the cellulose control. A reduction in bifidobacterial numbers was subsequently observed at 24 h for all fermentation conditions. No significant

difference in *Bifidobacterium* numbers was observed between the Fse extract and either control group. Culturable *Lactobacillus* were selected for using LBS agar plates with glacial acetic acid. A reduction in *Lactobacillus* numbers was recorded at every time point during the Fse fermentations. The number of *Lactobacillus* recovered reached their highest level after 5 h for the cellulose control ($1.6 \times 10^6 \pm 3.6 \times 10^6$) and FOS control ($1.2 \times 10^7 \pm 5.4 \times 10^6$). A reduction was recorded at each subsequent time point for cellulose and FOS (Fig. 3.2).

3.4.3 Short chain fatty acid analysis of fermentation samples.

Samples were taken at all time points (t = 0 h, 5 h, 10 h, 24 h, 36 h and 48 h) for short-chain fatty acids analysis. Analysis was carried out using Gas Chromatography – Flame Ion Detector (GC-FID) system. All mean values reported are minus the baseline values recorded at time 0 h, and are reported ± SE. For all fermentation conditions, approximately 90% of cumulative SCFA production took place during the initial 24 hours. During this time, total SCFA production in the control cellulose fermentation remained relatively evenly distributed, with 38.7% ± 4.0% of SCFAs being produced between 0 - 5 h, 20.4% ± 4.3% between 5 – 10 h and 20.1% ± 3.1% between 10 – 24 h. In contrast to this, more than half of total SCFA production (52.1% ± 7.5%) during the Fse extract fermentation occurred in the initial 5 h. A similar pattern was observed in the FOS fermentation, where 57.8% ± 5.2% of total SCFA were produced during the initial 0 - 5 h period (Fig 3.3, Fig 3.4). A significant increase ($p<0.05$) in total SCFA concentration was recorded for both the Fse extract and the FOS at all time points (t = 0 h, 5 h, 10 h, 24 h, 36 h and 48 h), in comparison with cellulose (Fig. 3.5). Total SCFA production was significantly increased ($p<0.05$) by 45.6% ± 12.7 with the FSE extract and 162.3% ± 5.1 with the FOS extract (Fig. 3.3). The primary SCFA produced was acetate, accounting for 47.8% ± 2.3 of Fse extract SCFA production, 51.7 % ± 2.7 for FOS, and 49.1% ± 4.3 for cellulose. Acetate concentration was significantly increased ($p<0.05$) with the Fse extract at 5 h, 10 h, 24 h and 36 h, in comparison with cellulose. Acetate production was significantly ($p<0.05$) between 0 – 5 h. (Fig. 3.6) Total acetate production for the Fse extract was increased by 41.9% ± 6.6 %, in comparison with cellulose. The second major SCFA produced was propionate (Fig. 3.7). A significant increase ($p<0.05$) in the total production of

propionate was observed for the Fse extract. Propionate production was increased by 137.2% ± 49.0 in comparison with cellulose. Propionate production was significantly increased ($p<0.05$) between 0 – 5 h. Propionate concentration was seen to be significantly higher ($p<0.05$) at all time points with the Fse extract, in comparison with cellulose. The third major SCFA produced was butyrate (Fig. 3.8). No significant increase in butyrate concentration or production was observed with the Fse extract at any point. Total production of butyrate was increased by 25.2% ± 12.5. with the Fse extract. A trend towards a reduction in overall BCFA production was observed in both the Fse extract and FOS fermentations indicating a shift in fermentation profile away from the degradation of proteins and amino acids (Fig. 3.9). The fermentation of FOS resulted in significant increases ($p<0.05$) in total SCFA concentration and production and significant increases in concentration and production of acetate, propionate and butyrate. The Fse extract fermentation had a significant effect on the molar ratio of total SCFA production and on production of the major SCFAs (acetate, propionate, and butyrate). The proportion of propionate produced during the fermentation was significantly increased ($p<0.05$), rising from 15% to 24% (total SCFA production) and from 18% to 26% (acetate, propionate, butyrate) (Table 3.2).

3.4.4 High throughput DNA sequencing of the 16s rRNA gene variable V4 region.

High through-put DNA sequencing was used to investigate the composition of the microbiota in an *ex vivo* model. The data were normalised and differences in the relative abundance between the initial time point and were compared with the cellulose control. Seven bacterial phyla were identified across the various samples taken; Actinobacteria, Bacteroidetes, Firmicutes, Lentisphaerae, Proteobacteria, Tenericutes, and Verrucomicrobia. Of these, Firmicutes were the most abundant accounting for greater than 50% of reads in each sample, followed by Bacteroidetes and then Proteobacteria (Fig. 3.12). At the phylum level, there was no significant change in relative abundance between the Fse extract or FOS in comparison with the cellulose control. A significant increase ($p<0.05$) in relative abundance was observed between Proteobacteria in the Fse extract and FOS (Fig. 3.13).

The major bacterial families present were *Bacteroidaceae* (Bacteroidetes), *Lachnospiraceae* (Firmicutes), *Ruminococcaceae* (Firmicutes), *Porphyromonadaceae* (Bacteroidetes), and *Prevotellaceae* (Bacteroidetes). At the family level, the Fse extract fermentation recorded a significant increase ($p<0.05$) in the relative abundance of *Porphyromonadaceae* (Fig. 3.14) in comparison with cellulose. For FOS, a significant increase in the relative abundance of the uncultured *Bacteroidales* family *S24-7 (*Fig 3.14) was observed in comparison with the cellulose control. There was no significant difference in changes of relative abundance at the family level between the Fse extract and FOS. There was no significant effect on the families *Bifidobacteriaceae* or *Lactobacillaceae* with any of the fermentations

The dominant genera of bacteria present throughout the fermentations runs were *Bacteroides*, *Lachnospiraceae* incertae sedis and another uncultured *Lachnospiraceae* genus, *Prevotella*, and an uncultured *Ruminococcacea* genus. At the genus level, the Fse extract caused a significant increase ($p<0.05$) in the relative abundance of an uncultured *Lachnospiraceae* bacterium (Fig. 3.17). At the genus level, the FOS fermentation recorded a significant increase ($p<0.05$) in the relative abundance of *Sutterella (*Fig. 3.16), *Christensenella* (Fig. 3.17), and significant decrease in relative abundance ($p>0.05$) with *Flavonifractor* (Fig. 3.17). There was no significant effect on the genera *Bifidobacterium* (Fig. 3.15) or *Lactobacillus* (Fig 3.17) with any of the fermentations

3.4.6 Measurement of Alpha (α) and Beta (β) diversity.

Alpha (α)-diversity was measured using Chao1 richness estimation, Shannon's index of diversity, Simpson index of diversity, observed species and phylogenetic diversity metrics were used to estimate α-diversity. Scatter plot analysis of alpha diversity revealed that supplementation with cellulose had no notable impact on alpha diversity measurement. However, a slight trend towards greater diversity was observed with the Fse extract for two of the fermentation runs. Beta (β) diversity was measured using an unweighted Unifrac distance matrix and visualised in a principle coordinate analysis plot. This visualisation showed that the samples from each vessel were clustered together at T0 and that supplementation with either the Fse extract or the FOS control had no notable effect on the changes in the microbial populations.

3.5 Discussion.

This study made use of an ex vivo faecal fermentation model to evaluate the prebiotic potential of a polysaccharide extract produced from *F. serratus*. This seaweed was chosen as data from a previous investigation (Chapter 2) indicated that an extract from this seaweed has bifidogenic properties. By consensus, the key characteristics that serve as the criteria for the classification of a compound as a prebiotic are the ability to resist gastric acidity and hydrolysis in the upper regions of the gastrointestinal tract and to be fermentable upon reaching the colon, leading to the selective growth and/or stimulation of a limited number of microbiota members [48, 49]. To satisfy the first of these conditions, it was important that the raw seaweed material was handled and treated in a way that would ensure an optimised prebiotic study. As such, the choice of the correct extraction method was of paramount importance. Solid-liquid extractions that use either hot/cold water and ethanol/methanol as solvents are commonly used for the collection of a variety of seaweed bioactive compounds [18] but sulphated polysaccharides from seaweeds are generally extracted using hot water or dilute acid/alkali [50]. While these methods allow for the extraction of seaweed polysaccharides, they also extract algal compounds that would not be desirable to have in a prebiotic candidate such as polyphenolic compounds that may exhibit antimicrobial activity [51], or simple monosaccharides such as glucose and fructose which could cause false positives. Previously utilised seaweed extracts were basic extracts where cold water was used as the extraction solvent. No post-extraction steps were taken to refine them and, thus, an extract containing a multitude of biological active components was produced. Here, the primary extraction was carried out using dilute hydrochloric (HCl) acid (0.1 M) at 70 °C. The use of hot dilute acid, rather than ethanol/methanol or cold water, aided in the extraction of the seaweed's structural polysaccharides located in the algal cell wall. Protons from HCl interfere with the hydrogen bonds between the various polysaccharides, releasing them into solution resulting in an increased yield [50]. After the primary extraction, ethanol was used to precipitate the carbohydrate content out from the crude extract, while simultaneously removing large quantities of salts and non-polar materials. Soluble seaweed sugars and polysaccharides interact extensively with water molecules when in solution. The use of an organic precipitant, such as ethanol, interrupts these interactions allowing

for the separation of the sugars and polysaccharides from the solutions. The higher the alcohol concentration the more the solubility of the polymers in the solution [52]. After centrifugation at high speed, the supernatant was removed and discarded and the pelleted precipitate, containing the seaweed sugars, was collected and stored after being freeze-dried. Although ethanol precipitation can effectively isolate carbohydrates from the non-carbohydrate bulk, simple sugars and other bioactive components can remain in the extraction mass. To remove these from the extract, several rounds of size exclusion dialysis, with a molecular cut off point of 1 kDa, were employed. The final post extraction step was an *ex vivo* simulated gastric digest, in order to mimic gastric transit. Breakdown products from the simulated digest were removed by a final size exclusion dialysis (1 kDa) step. The *F. serratus* polysaccharide extract, henceforth termed the Fse extract, was then blast frozen and freeze died before storage.

The hydrolysis of polysaccharides to component sugar units is a common and crucial step in structural analysis. Soluble polysaccharides can be broken down to their component monosaccharides in TFA [53]. Seaweed polysaccharides are polymers of simple sugars linked together by glycosidic bonds [54] and thus, from knowing the basic monosaccharide building blocks, the parent polysaccharide can be reasoned. Prior to treatment with TFA, the Fse extract had no measurable concentration of the monosaccharides glucose or galactose. This was expected as free glucose and galactose, and other low molecular weight components, would have been removed following the 1 kDa size exclusion dialysis. Following treatment with TFA, appreciable amounts of both glucose (84.0 µg/ml) and galactose (86.1 µg/ml) were detected within the degraded Fse. Laminarin is a β-glucan, which mainly consists of β-1,3-D-glucopryanose residues [55]. B-glucans are naturally occurring polymers of glucose that are produced by a variety of plants such as oats, barley and seaweed [56]. A fucoidan, described by Bilan et al. [57], consisting of L-fucose, sulfate and acetate with small amounts of xylose and galactose was previously isolated from *F. serratus*. As the levels of glucose and galactose detected in the degraded extract were at an approximately equal concentration and that galactose is a minor component of fucoidan, we hypothesize that the parent polysaccharides present in the Fse extract of prebiotic interest include fucoidan and laminarin with fucoidan being present at a higher concentration than laminarin. Both fucoidan and laminarin have been

demonstrated to be resistant to salivary, gastric, pancreatic and intestinal enzymes [58, 59]

The human proteome in the GIT has a limited range of the glycoside hydrolases for the digestion of complex dietary plant polysaccharides. It is the microbiota, which synthesise a large number of these enzymes, that allows us to convert dietary carbohydrates to short-chain fatty acids, principally acetate, propionate and butyrate [60]. Bacterial fermentation in the gut comprises several different metabolic pathways. The fermentation end-products of one species can serve as a growth substrate for another. In this manner, some microorganisms benefit from substrates which they are not able to utilize directly [61]. Fermentation of FOS resulted, as expected, in the largest increase in total SCFA production. The FOS was quickly fermented, by the *ex vivo* microbial populations, giving rise to significantly increased levels ($p<0.05$) of total SCFAs as well as significantly increased levels of total acetate, propionate and butyrate at all recorded time points in comparison with the cellulose control. This result indicates that the anaerobic faecal fermentation model was appropriately designed and implemented for short-chain fatty acid analysis and prebiotic investigation. Significant increases in SCFA production were also observed for the Fse fermentation but to a lesser extent than the FOS control. This observation agrees with a study by Deville et al., [20] in which laminarin isolated from *Laminaria digitata* exhibited an effect like the FOS control but in comparison with FOS, the Fse extract is a crude one containing carbohydrates of differing molecular weights, varying degrees of polymerisation and uncertain fermentation potential. The physical and chemical properties of carbohydrates affect which members of the microbiota will be involved in its fermentation. This in turn affects both the quantity and type of SCFAs produced. Most SCFA production took place early in the fermentation indicating that the polysaccharides (fucoidan, laminarin,) contained within Fse extract were readily fermented by the *ex vivo* microbiota. As cellulose is poorly metabolised by the microbiota, and no additional carbohydrate source was supplied during the fermentation, observed increases in SCFA production in the control vessels can principally be attributed to the fermentation of dietary substances carried over from the composite faecal sample. The three principal SCFAs (acetate, propionate and butyrate) alone accounted for more than 85% of the total SCFAs produced in the cellulose fermentation and more than 90% in the Fse and FOS fermentations.

Significant increases ($p<0.05$) in the production of both acetate (1.4-fold increase) and propionate (2.4-fold increase) were observed with the Fse fermentation. Non-significant increases in butyrate concentrations were also observed for the Fse fermentation. The results of this investigation also broadly agree with a similar study involving batch fermentations with low molecular weight polysaccharide derivatives of alginate and agar as the test compounds. There, significant increases of acetate and/or propionate were recorded, while all seaweeds produced very low or negligible levels of butyrate [10]. While it is understood that all dietary fermentable carbohydrates that reach the colon have the potential to produce butyrate, not all fermentable substrates are equally butyrogenic. Resistant starch and oligofructose, for example, are associated with greater production rates of butyrate as a proportion of the total SCFA produced than pectin or alginate, which is a major polysaccharide in brown seaweed. [62].

Chemical and physical properties also influence which members of the microbiota will be involved in its fermentation. Studies have shown that SCFA production in the proximal and distal colon is in the order of acetate > propionate = butyrate, in a molar ratio of approximately 60:20:20 [63] with the ratio varying among individuals because of difference in their microbiota and in types and amounts of carbohydrates consumed in the diet. Forty-eight hours after inoculation of the fermentation vessels, the acetate/propionate/butyrate ratio of the cellulose control (without the supplementation of any carbohydrate) was 57:18:26 in approximate agreement with other reported values. The acetate: propionate: butyrate ratio of the Fse containing fermentation was observed to be 53:26:21, indicating a significant alteration ($P<0.05$) of the relative amount of propionate produced as well as a corresponding decrease in the relative production of both acetate and butyrate. Studies dealing with the fermentation of brown seaweeds are contentious [64]. A fermentation study involving laminarin reported significantly increased levels of both butyrate and propionate in comparison to glucose, which was used as a non-prebiotic control [58]. The microbial fermentation of indigestible carbohydrates in the gut is the main source of propionate available in the body [65]. Propionate production is associated with the presence of greater amounts of β-glycosidic bonds as demonstrated by the fermentation of polysaccharides such as laminarin [58]. Propionate is a precursor for intestinal and hepatic gluconeogenesis and has shown several health promoting effects

that include anti-lipogenic, cholesterol-lowering, anti-inflammatory and anti-carcinogenic activities [66]. Furthermore, as obesity levels continue to rise worldwide, there has been much interest recently in the role that propionate has in enhancing satiety. The selective increase of colonic propionate levels in humans, through the consumption of inulin propionate ester, has been shown to regulate appetite, reduce hepatic and intra-abdominal visceral fat deposits and reduce body weight in overweight adults [67]. Recent proteomic research indicates that some of the effects of propionate at the cellular level differ from the action of butyrate. In human colon cancer cells and neutrophils, the anti-proliferation capability of SCFA has been associated with the inhibition of histone deacetylase. Butyrate is viewed as the most effective inhibitor, with propionate being regarded as less potent. Acetate was seen not to harbor inhibitory activity [68]. The SCFA receptors FFAR2 and FFAR 3 are likely to mediate some of the actions of propionate. In a study carried out with mice transplanted with Bcr-Abl-transfected BaF3 cells, who received inulin-type fibres in their drinking water, both acetate and propionate were shown to reduce BaF3 cell proliferation. Propionate is one of the most potent endogenous FFAR2 ligands and FFAR2 is highly expressed by BaF3 cells [33, 69]. Further, both FFAR2 and FFAR3 are expressed in the intestine and colocalise with a subset of enteroendocrine cells in the mucosal epithelium that express Peptide YY (PYY). PYY and other peptide hormones secreted by enteroendocrine cells, such as Glucagon-like peptide 1 (GLP-1) and Glucose dependent insulinotropic polypeptide (GIP), are key modulators of energy homeostasis and glucose metabolism. Propionate is believed to be the most potent endogenous agonist for both FFAR2 and FFAR3. A study by Chambers et al. [67] demonstrated that propionate significantly stimulated the release of PYY and GLP-1 from human colonic cells. An increase in propionate levels in the colon could lead to an anti-obesity effect on humans. Even a small habitual increase in energy intake of just 50-100 kcal/day can lead to gradual long-term weight gain, with the reported average weight gain for an adult being 0.3-0.8 kg/year. A major challenge for public health agencies is the development of effective strategies that can prevent the increased prevalence of obesity. Interventions, that can be safely applied at the population level to reverse this minor energy imbalance and prevent weight gain throughout life, would therefore have substantial benefits for public health. Increasing propionate production by the colonic microbiota through dietary intervention would

be an attractive prospect in preventing overeating, increasing post prandial satiety and maintaining good general health.

DNA sequencing and other culture-independent technologies do not rely on cultivation in the laboratory but target nucleic acids directly to better study changes in the microbial populations present in specific niche environments [70]. The sequencing analysis of the 16s rRNA gene amplicon is a widely used technique for studying microbial communities in samples taken from a variety of different sources such as soil, blood and food [71]. The nine variable 16s rRNA gene regions are flanked by conserved regions in most bacteria and can be used as targets of PCR primers with near-universal bacterial specificity [72]. In this study, the V4 region of the 16s rRNA gene was targeted to examine the effect that the Fse extract had on the *ex vivo* microbial community. This variable region has been shown to be most efficient in identifying microbial populations in samples with high levels of sequence diversity [73].. The production timeline for SCFAs is also indicative of how well the Fse extract and the experimental controls were fermented by the *ex vivo* microbiota. Both the Fse extract and the FOS control were quickly and efficiently utilised by the endogenous microbiota with 57.002% ± 5.453 and 61.548% ± 4.668 of total SCFA, respectively produced after the first five hours of the fermentation. This implies that the Fse extract has prebiotic potential, however, three biological repeats lack the statistical power to provide a definitive analysis on the effect of seaweed polysaccharides, this is the first time that a processed polysaccharide-rich extract from *F. serratus* has been investigated in this manner.

Global stimulation of the gut microbiota would be detrimental to host gut health as it would allow for the proliferation of pathogenic species and a corresponding build-up of toxic metabolites. A putative prebiotic would need to have a selective effect on only the beneficial members of the gut microbiota. Culture independent 16s rRNA analysis has indicated that the two most abundant phyla in adults are the Bacteroidetes (normally between 10-50%) and the Firmicutes (up to around 75%). The dominant Firmicutes species mainly belong to the families *Lachnospiraceae* and *Ruminococcaceae*. Members of the phylum Actinobacteria, especially the genus *Bifidobacterium* can also be present in large numbers in the normal healthy adult colon (normally up to 10%), but have often been underestimated by 16s rRNA analysis if the correct primers are not used [74]. The classical view of a prebiotic would be that

its fermentation in the gut would result in the stimulation of *Bifidobacterium*, *Lactobacillus* and a few other beneficial members of the microbiota.

Supplementation of the fermentation vessels with the Fse extract did not have a widespread effect on the microbiota in the *ex vivo* model. An apparent selective stimulation of certain propionate and acetate producers, in agreement with observed trends in SCFA production, was observed. To understand and control SCFA formation by the colonic bacteria, knowledge of the phylogenetic groups and pathways which have roles in the formation of each acid is needed. The three phyla Bacteroidetes, Firmicutes and Actinobacteria are the most abundant in the intestine. The phylum Bacteroidetes mainly produces acetate and propionate, whereas the Firmicutes phylum has butyrate as its primary metabolic end product [75]. This information is currently available for butyrate but is lacking for propionate. Three different biochemical pathways are known for propionate production by the microbiota; the succinate pathway, the acrylate pathway, and the propanediol pathway. The most common metabolic pathway for propionate biosynthesis amongst the microbiota (*Bacteroides, Phascolarctobacterium, Dialister, Veillonella*) is the succinate pathway. Through this pathway, pyruvate is converted to oxaloacetate from pyruvate which in turn is converted into succinate, succinyl-CoA, propionyl-CoA and finally into propionate. *Propionibacterium* spp. is another common propionate producer that uses the succinate pathway. The acrylate pathway for propionate production has been characterised in the soil bacterium *Clostridium propionicum*, and the corresponding genes have been recently described. It is possible to distinguish the succinate pathway from the acrylate by incubation with stable isotope-labelled substances.

Finally, several bacteria are known to produce 1,2-propanediol from deoxy sugars such as fucose and rhamnose, or via different pathways from dihydroxyacetonephosphate or lactate. In some bacteria, 1,2-propanediol can be further metabolised to propionate or propanol [65, 69, 76]. There was a marked increase in the relative abundance of the propionate-producing family *Veillonellaceae* [77], the genus *Parabacteroides* that is known to produce both propionate and acetate and the family *Erysipelotrichaceae* which is peripherally related to the butyrate-producing superfamily *Lachnospiraceae*. Members of the family *Veillonellaceae* have an interesting metabolism in that they are unable to ferment carbohydrates including glucose but grow well anaerobically on lactate, pyruvate and malate [78]. Increases in

the relative abundance of this family would likely be because of bacterial cross-feeding. *Megasphaera,* a member of the family *Veillonellaceae,* can utilize lactate and convert D-Lactate to propionate [79]. A member of the family *Alcaligenaceae, Parasutterella excrementihominis,* isolated from human faeces was shown to produce trace amounts of propionate as an end product of metabolism [80]. Interestingly, both the Fse extract and the FOS control had a negative effect on levels of *Bacteroides* and *Prevotella,* (two major propionate producers), *Faecalibacterium* and the previously mentioned family *Lachnospiraceae* which belong to the major butyrate producing Clostridial cluster IV of the Firmicutes [81, 82]. In agreement with the plate count data for the probiotic bacteria, the Fse extract had no positive impact on the relative abundance of bifidobacteria or lactobacillus populations. A substantial increase in the mean relative abundance of bifidobacteria was seen with the FOS fermentation although no impact was seen on the abundance of lactobacilli.

Scatter plot analysis of Alpha diversity indicated a slight trend towards greater diversity of species for the *F. serratus* fermentation. This infers that the extract had a wider stimulatory effect than either the FOS or cellulose control. This could be because of the presence, in the *F. serratus* extract, of a wider spectrum of oligo – and polysaccharides that have different glycosidic linkages which more bacterial species can degrade. Unifrac β-diversity analysis indicates the extent of similarity between microbial communities [83]. Unweighted PCoA (Principle co-ordinate Analysis) plots indicated a clear separation between the fermentation communities based on run rather than treatment condition. Supplementation with either the Fse extract or the FOS control had no notable effect on the similarity between their respective *ex vivo* populations and that of the cellulose control.

Most intestinal bacteria are saccharolytic which means that, in general, they hydrolyse dietary polysaccharide, first to oligosaccharides of a lower molecular weight and then to monosaccharides using a diverse range of carbohydrate modifying enzymes and transportation systems. Bifidobacteria, for example, are able to use a diverse range of dietary carbohydrates that are not digested in the upper regions of the GIT and reach the colon intact [28]. As cellulose is poorly metabolised by the gut microbiota [84], substantial bacterial proliferation would not be expected in a fermentation vessel where cellulose was the only available carbohydrate source. The only energy sources of note available for fermentation in the negative control vessels

would be from the inevitable crossover of small amounts of unused carbohydrates in the added composite faecal slurry. In contrast, both the polysaccharide rich Fse extract and the FOS control contain complex carbohydrates that are available for the microbial community to use as an energy source for growth. Serial dilutions of collected fermentation sample were carried out at 0 h, 5 h, 10 h, and 24 h in maximum recovery diluent to determine *Bifidobacterium* and *Lactobacillus* numbers (Log_{10} CFU/ml). The respective agar plate data for the enumeration of bifidobacteria and lactobacilli showed that the Fse extract had no significant impact (p-value <0.05) on the numbers (Log_{10} CFU/ml) of either. However, a noteworthy, non-significant, effect on bifidobacteria numbers was observed with FOS between 0 h and 10 h.

The ability of bifidobacteria to metabolise complex carbohydrates has allowed them to inhabit a unique ecological niche. From this, the host gains energy by absorbing SCFAs while the bifidobacteria are protected in a safe, anaerobic environment and are supplied with large quantities of glycans. Analysis of the genome of *B. breve* UCC2003 revealed the presence of a *fos* operon that encodes for a putative permease, a conserved hypothetical protein and a β-fructo-furanosidase, all of which have involved in the metabolism of short-chain FOS [85]. Despite this, FOS had little or no observed effect on numbers of bifidobacteria. The ability to stimulate the growth of beneficial members of the gut microbiota is an important part of the prebiotic definition as currently understood. In the years to come, it is likely that new subclasses, with tightly defined criteria of activity, will be established.

The apparent difficulty that intestinal bacteria had in fermenting the Fse extract was not completely unexpected. While alginate and laminarin are fermented by some strains of *Bacteroides* and *Clostridium*, most intestinal strains of microflora cannot ferment fucoidan. The breakdown of the α- (1-3) bonds in fucoidan is done by fucoidanases, enzymes that are only found in marine bacteria and mollusks. Although *Bifidobacterium* cannot utilise laminarin it is reported that degraded products from laminarin produced by *Clostridium ramosum*, a general human intestinal bacteria, are utilised by *Bifidobacterium* strains [59, 86]. The FOS fermentation resulted in large increases in production of all three biologically important SCFAs and in *Bifidobacterium* populations indicating that the experimental design for this prebiotic potential was fundamentally sound. More detailed studies on the prebiotic potential of

F. serratus polysaccharides, as well as seaweed derived polysaccharides in general, should be carried out by fractionating the algal polysaccharides into different molecular weight bands and then testing each M.W. fraction separately or in combination for prebiotic activity. Currently, prebiotics have been tested to stimulate *Bifidobacterium*, *Lactobacillus* and a small number of other organisms such as *Eubacteria* and *Faecalibacteria*. While stimulating the actual growth of select members of the microbiota is clearly important in terms of being a prebiotic, it is only one part of the prebiotic definition, as currently stated, coupled with the stimulation of bacterial activity. The definition, as quoted in the introduction, is an ever evolving one as more and more knowledge becomes available regarding the inner workings of our gut and the complex relationships that exist amongst its resident microbiota and their environs. In time, new classes or subcategories of prebiotic may be established that have well-defined modes of action and may supersede our current definitions of prebiotics and expand upon the criteria currently employed in their description.

3.6 Conclusion.

In this chapter, we have demonstrated that the Fse extract was successfully able to tolerate a simulated gastric digestion where it was subjected to both salivary and gastric digestive enzymes as well as acidic conditions mimicking the stomach environment. When the Fse extract was added to an *ex vivo* faecal fermentation system, the representative microbiota successfully used the algal polysaccharides as fermentation substrates as indicated by noticeable increases in SCFA production especially propionate and acetate. Fermentation of the extract also resulted in non-significant increases in butyrate concentration. Fermentation of the Fse extract did not stimulate increased growth of *Bifidobacterium* or *Lactobacillus* or significantly alter their relative abundance within the representative microbiota. The results indicate that the fermentation of the Fse extract did not bring about a desired prebiotic effect as outlined previously, possible because of the low fermentability of *F. serratus* fucoidan. Interestingly, a similar result was obtained for the known prebiotic FOS. During the FOS fermentation, there was also no significant effect on *Bifidobacterium* or *Lactobacillus* in terms of relative abundance or in recovery of culturable strains. This could simply be because the extract concentration used in the study was too low for a prebiotic effect to be evident, or that the absence of the required enzymes for the breakdown of seaweed polysaccharides such as fucoidan (fucoidanases), reduced the overall fermentability of the Fse extract. Further, it is known that brown seaweeds possess components, such a phlorotannins, that have potent anti-microbial properties. The carryover of such compounds from the extraction may have reduced the overall effectiveness of the extract from a prebiotic point of view.

While the Fse extract, prepared as part of this study did not exhibit all the prebiotic markers an expected increase in the production of SCFA, especially propionate, is a positive outcome in terms of promoting overall gut health and in combating obesity through enhancing satiety. Also, as the extract was shown to withstand gastric digestion, *F. serratus* would be an excellent source of dietary fibre as part of a healthy balanced diet.

3.7 References.

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Figure 3.1 Carbohydrate analysis of the *F. serratus* polysaccharide extract pre-treatment and post-treatment with trifluoroacetic acid (TFA).

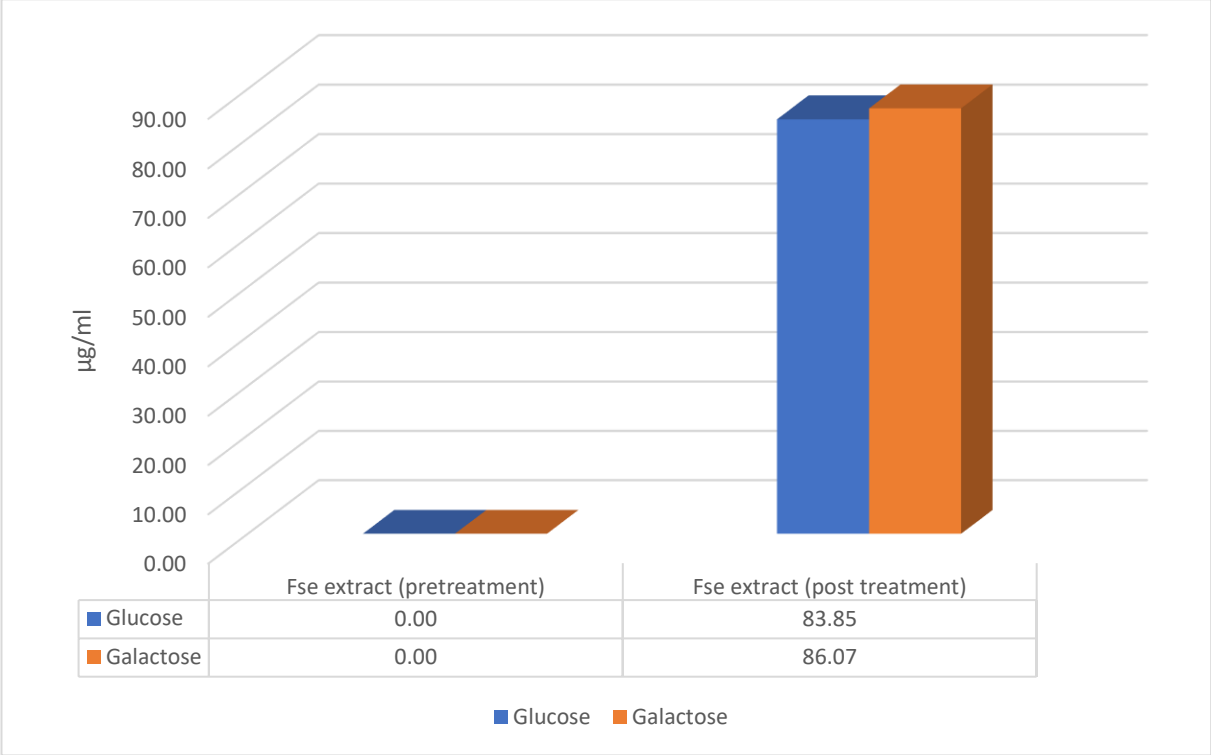
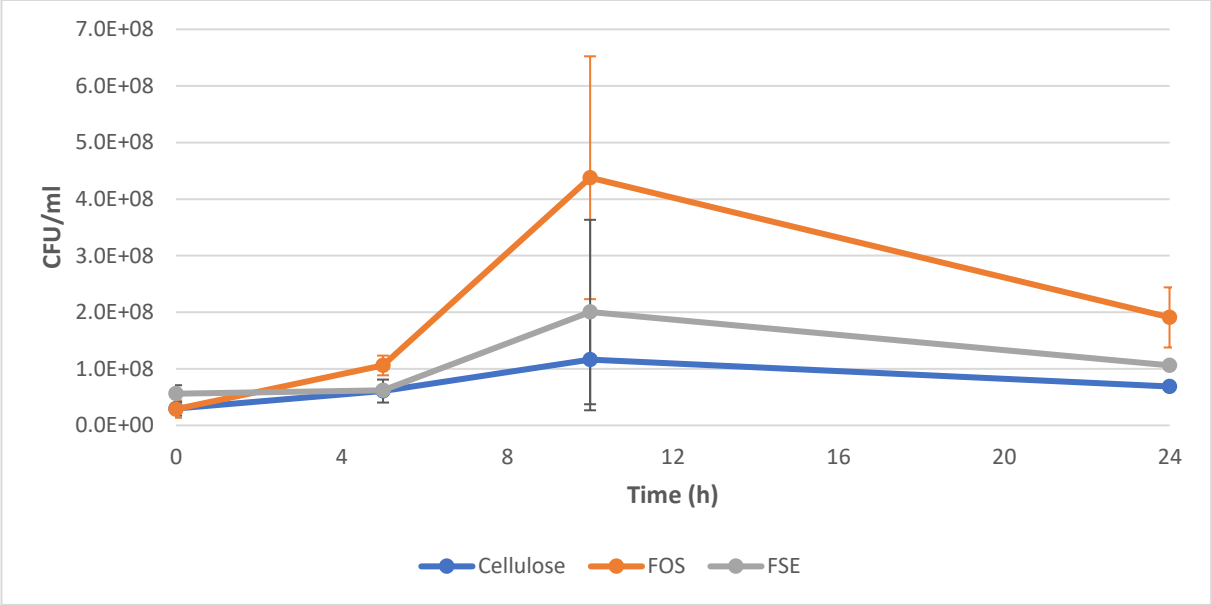


Figure 3.2 Effect on culturable (a) *Bifidobacterium* and (b) *Lactobacillus* recovered. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

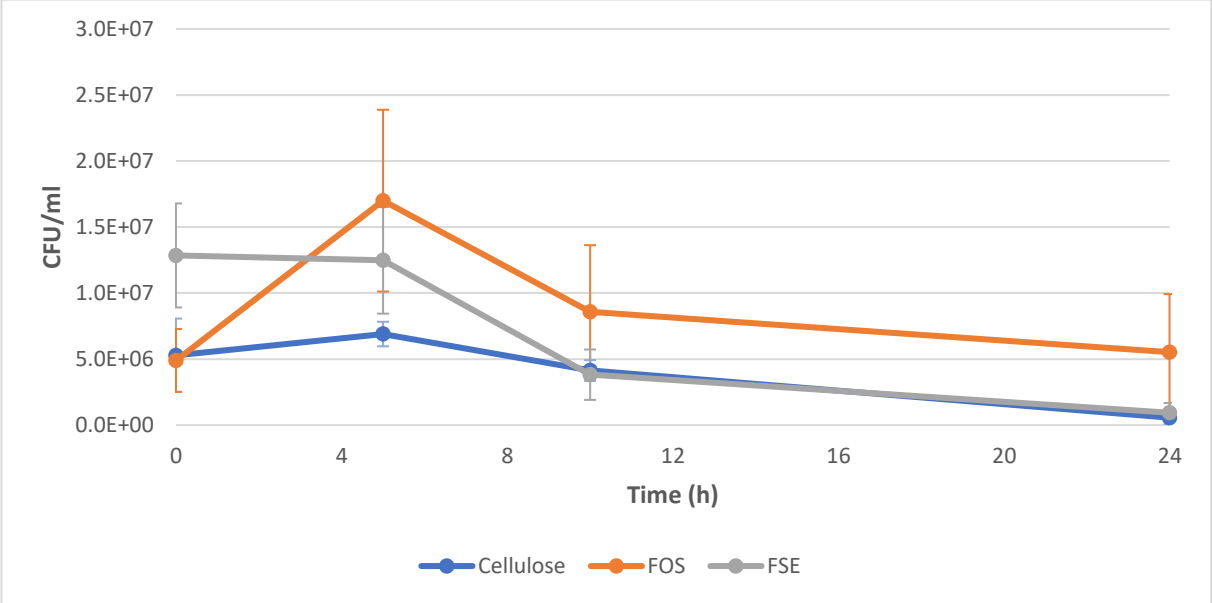
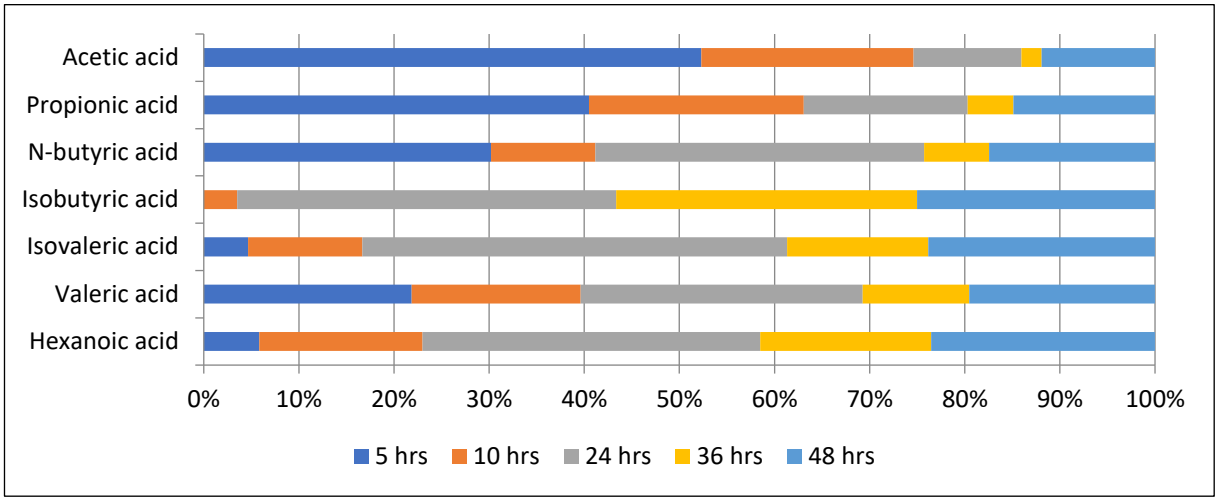


Figure 3.3 SCFA production timeline. The timeline of SCFA production for (a) cellulose, (b) FOS and (c) *F. serratus* extract**.**

(a)



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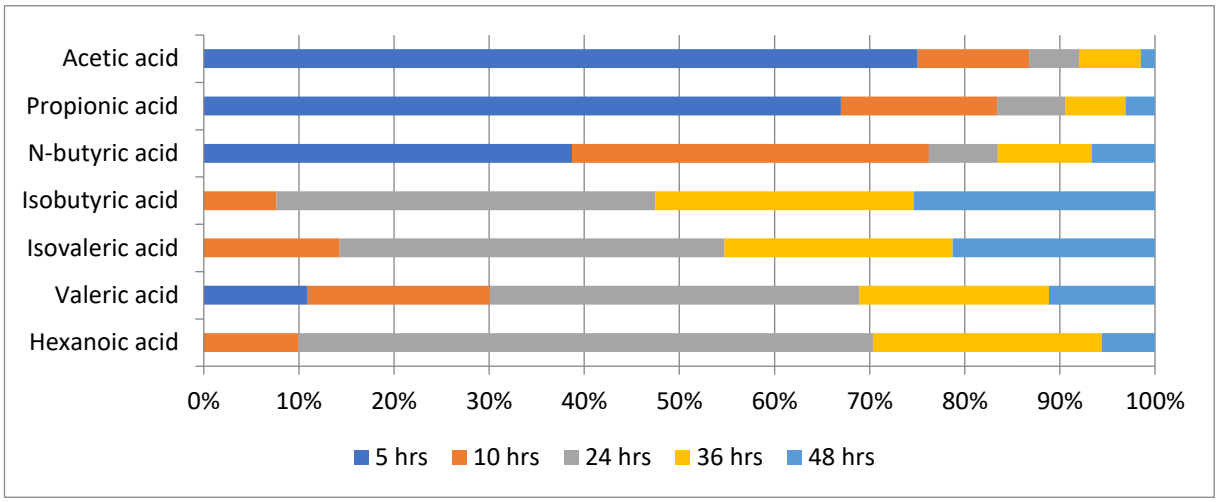


(c)

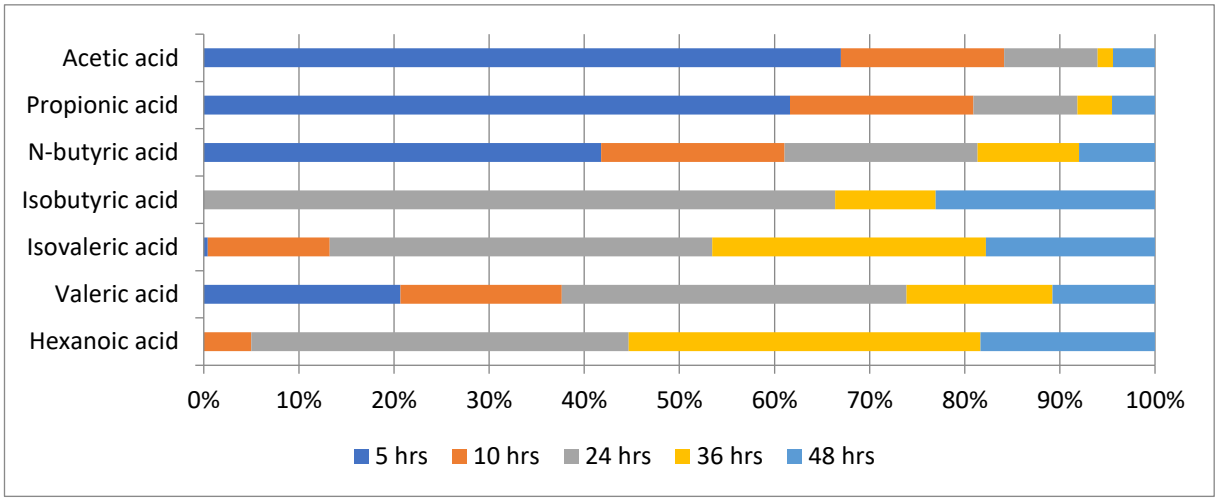
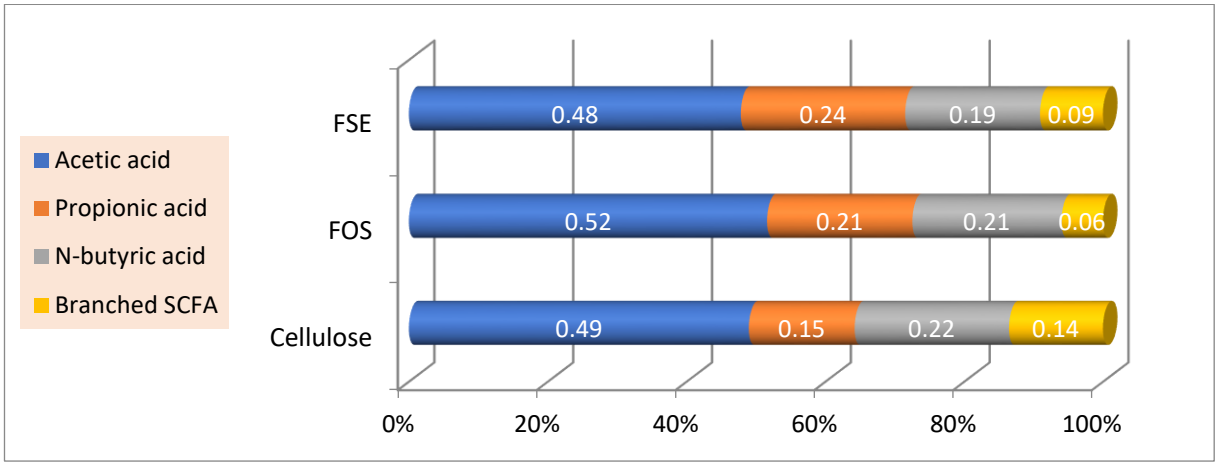
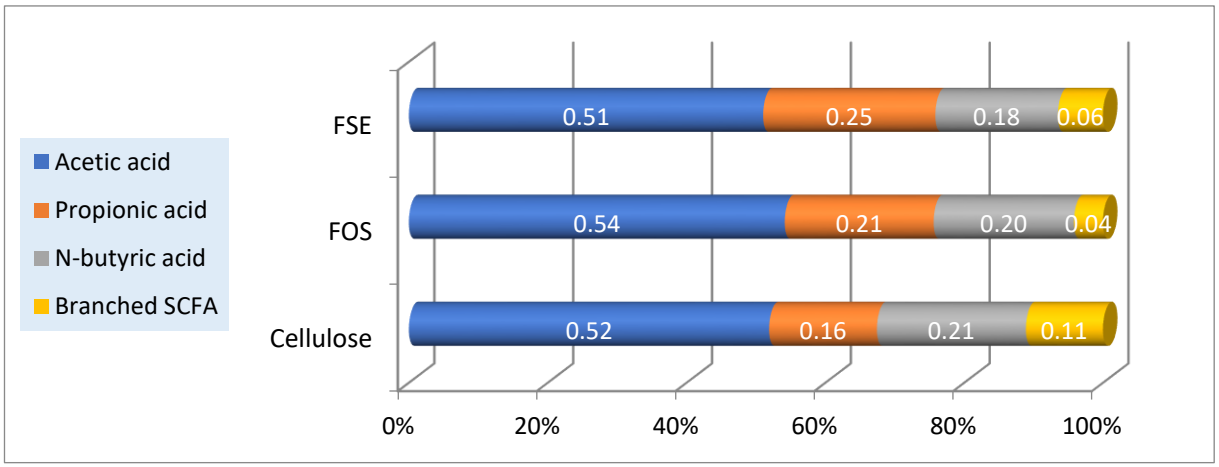


Figure 3.4 The percentage breakdown of total SCFA production. Breakdown of SCFA production (a) total, (b) between 0-24 h and (c) between 24-48 h. Date represents mean values.

(a) Total SCFA production



(b) SCFA production 0 – 24 h



(c) SCFA production 24 – 48 h

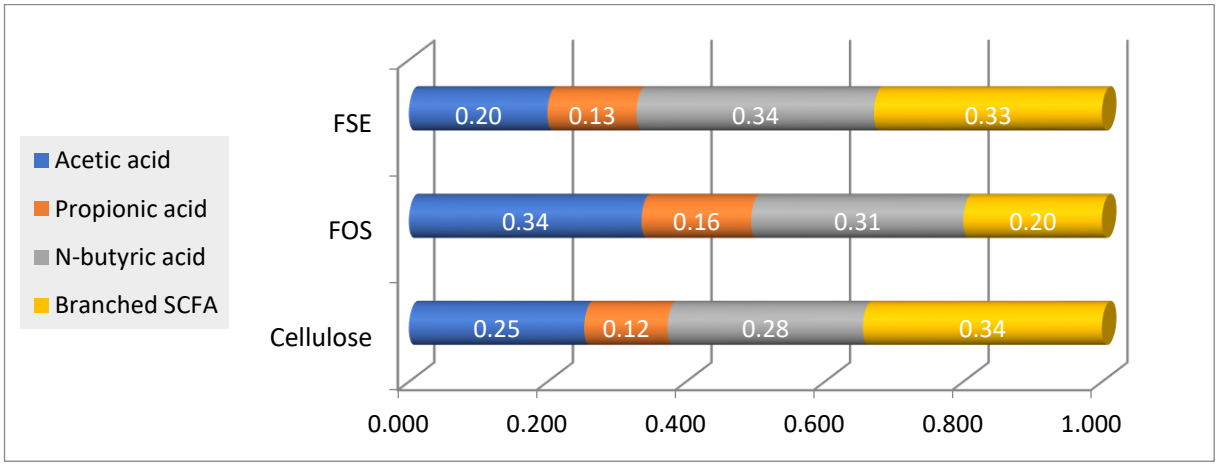
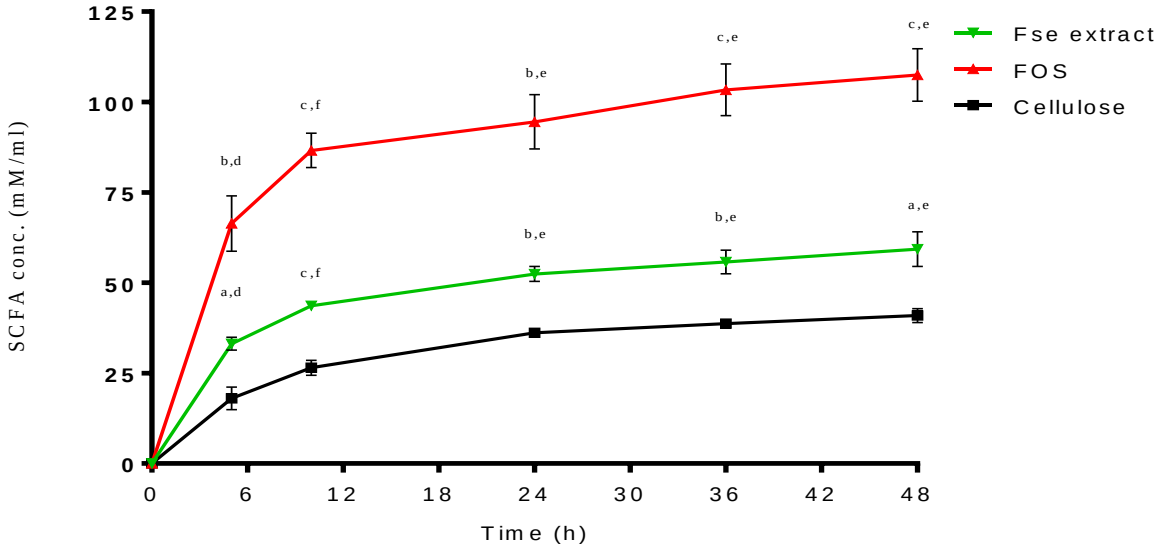


Figure 3.5 The effect of the *F. serratus* extract and FOS on (a) total SCFA concentration and (b) total SCFA production per time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

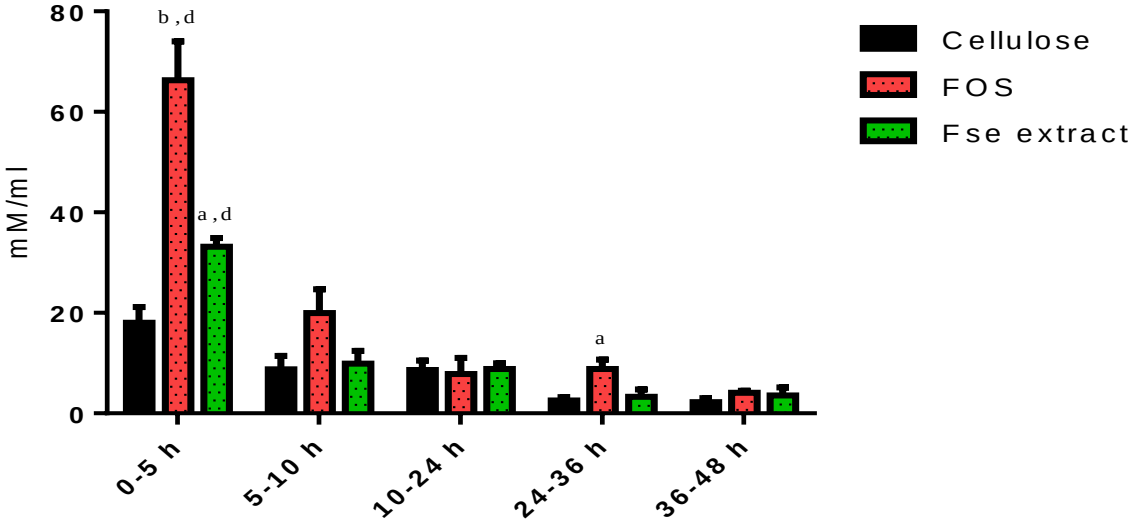
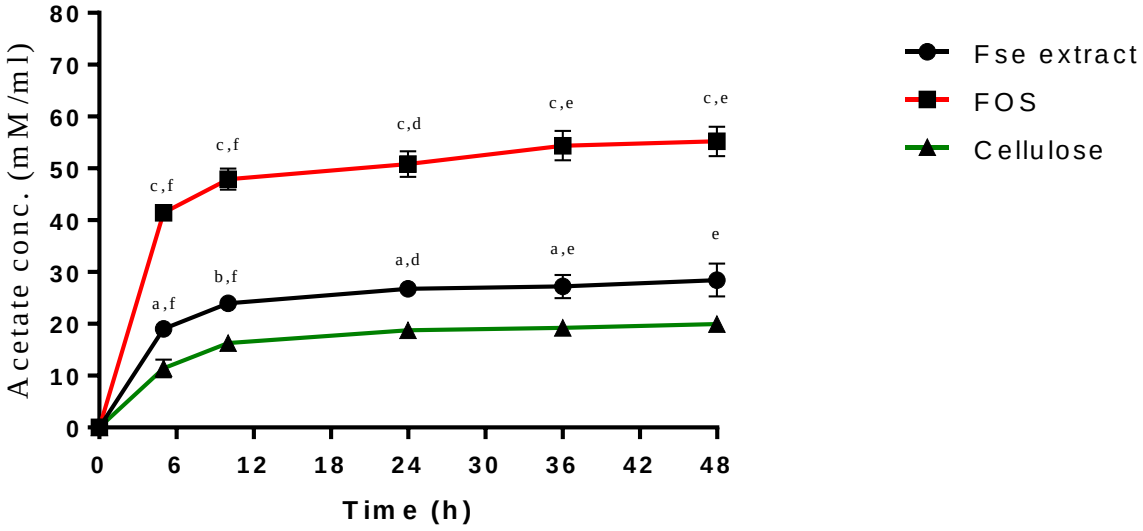


Figure 3.6 Acetate production. The effect of the *F. serratus* extract and FOS on (a) acetate concentration and (b) acetate production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test)

(a)



(b)

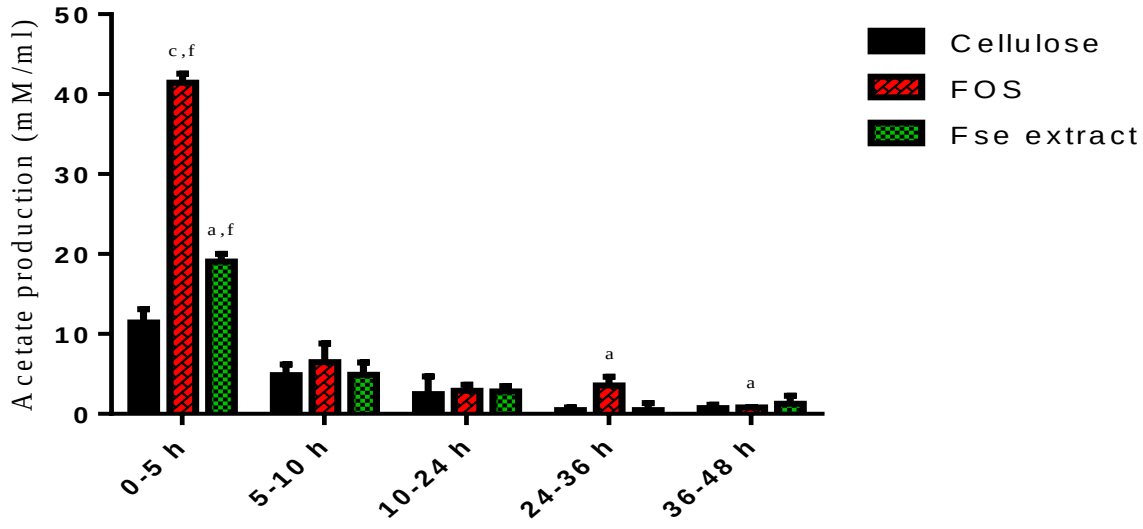
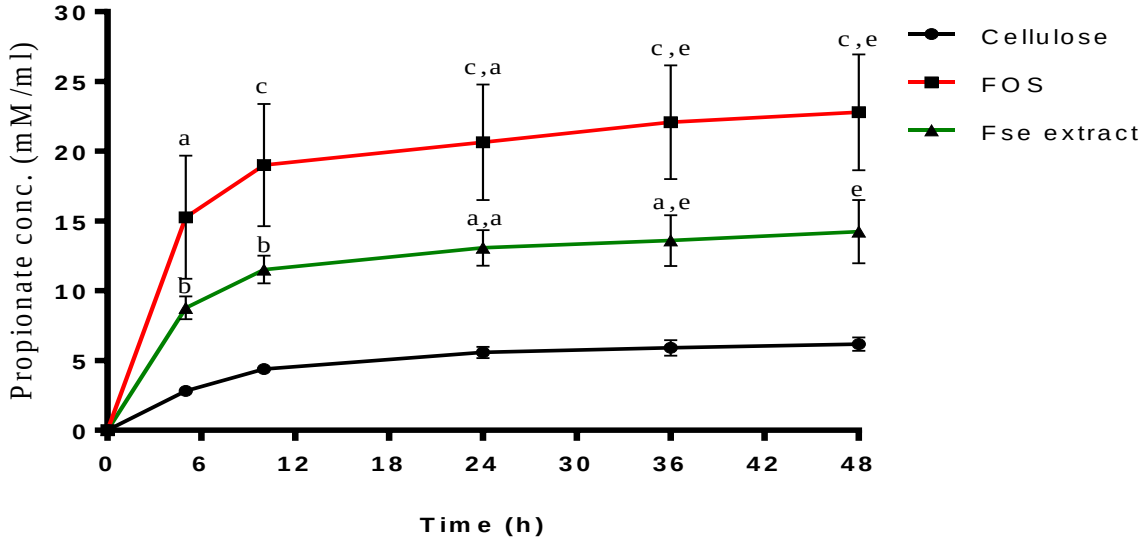


Figure 3.7 Propionate production. The effect of the *F. serratus* extract and FOS on (a) propionate concentration and (b) propionate production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

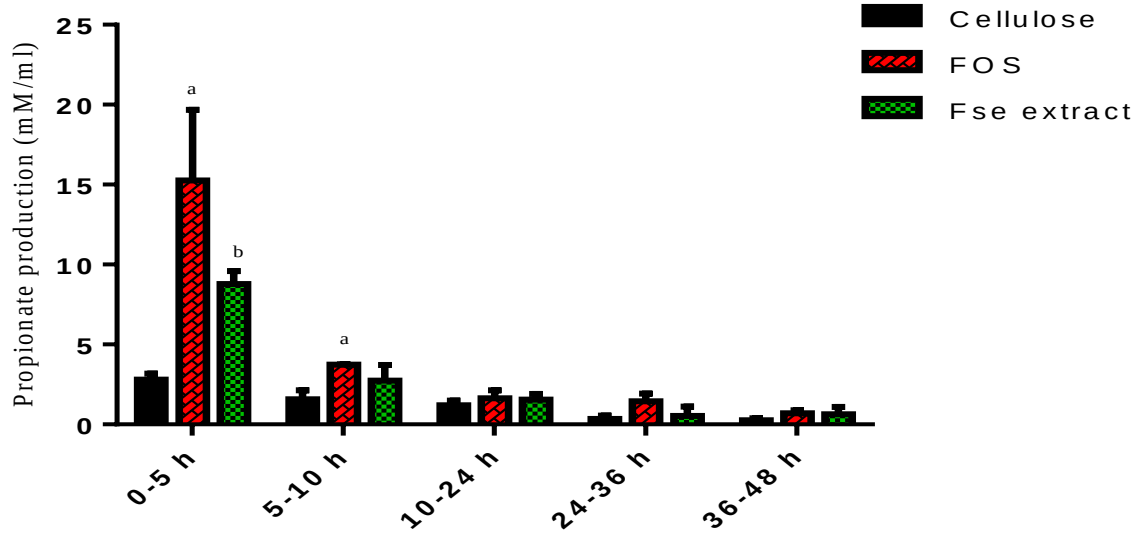
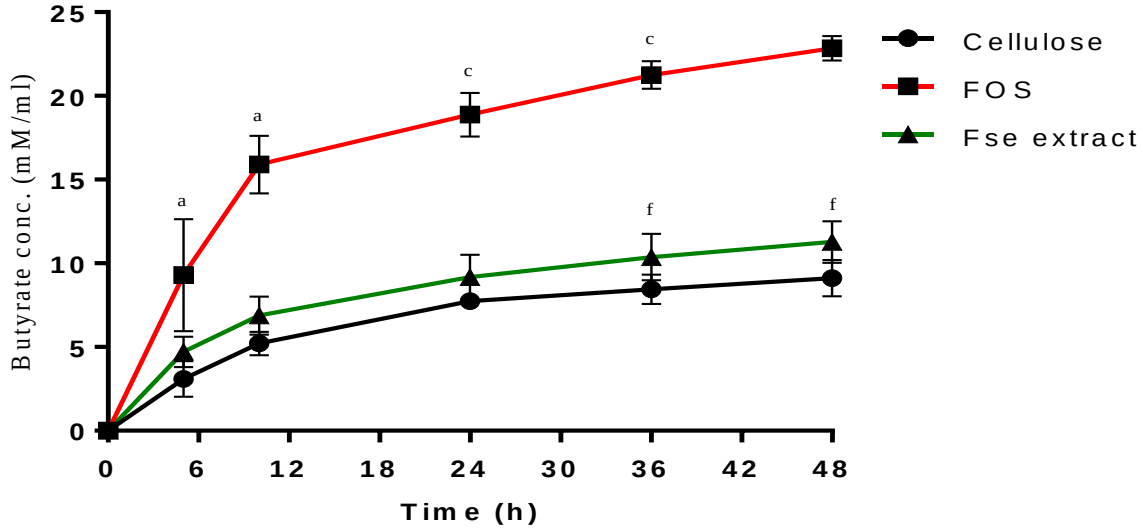


Figure 3.8 Butyrate production**.** The effect of the *F. serratus* extract and FOS on (a) butyrate concentration and (b) butyrate production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test)

(a)



(b)

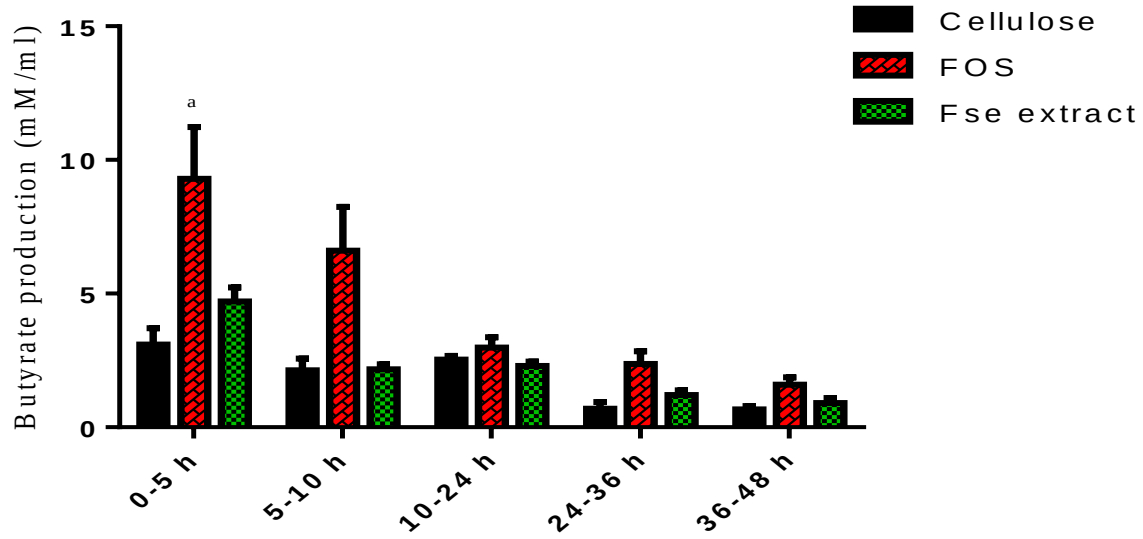
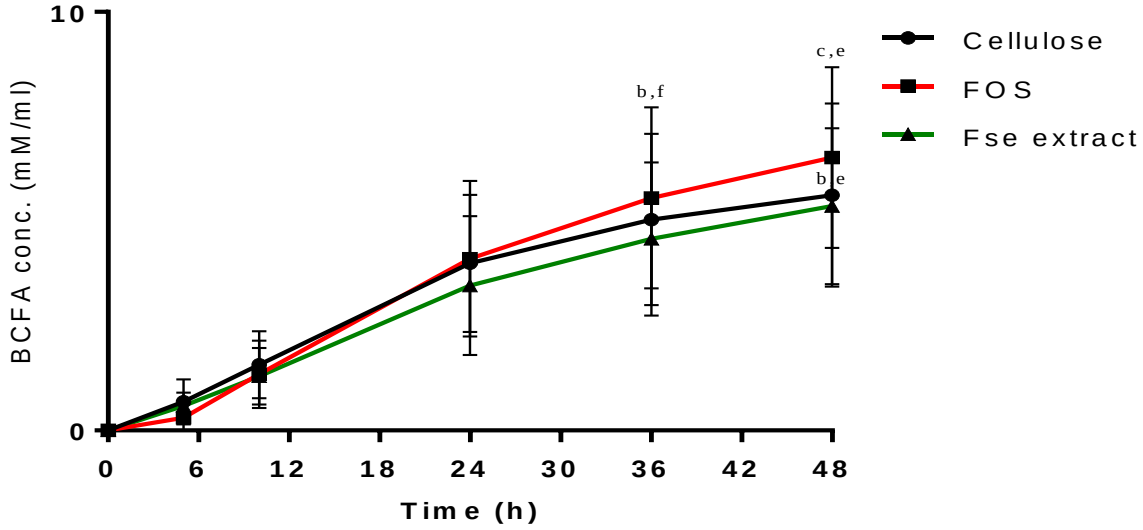


Figure 3.9 Branched-chain fatty acid production. The effect of the *F. serratus* extract and FOS on (a) BCFA concentration (b) BCFA production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test)

(a)



(b)

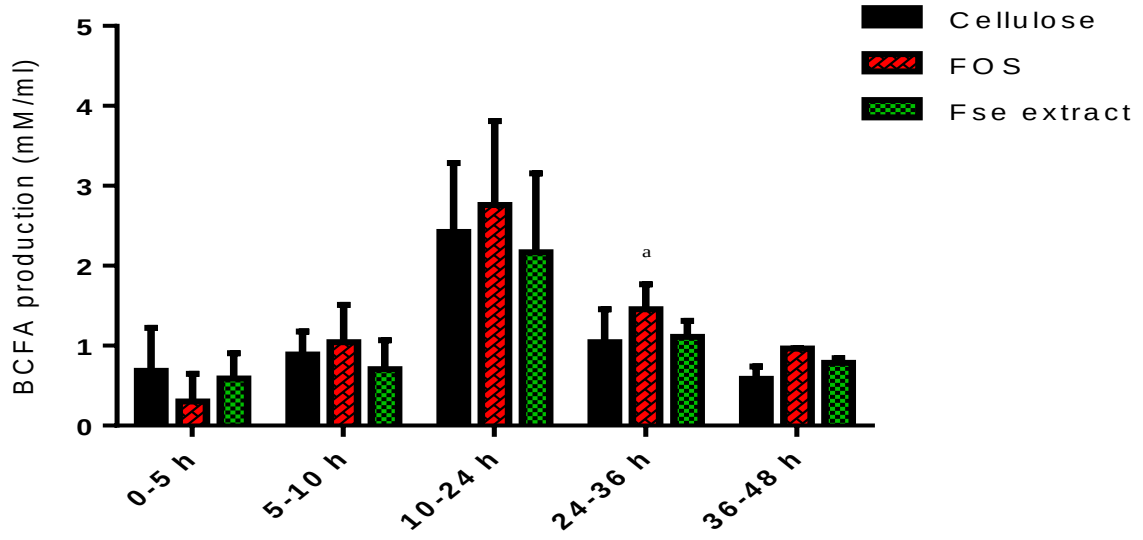


Table 3.1. The fermentation of the *F. serratus* extract resulted in significant changes in (a) the molar ratio of acetate: propionate: butyrate: BCFAs, and (b) the molar ratio of acetate: propionate: butyrate. (a = P<0.05) relative to cellulose.

(a)

	Acetate:	Propionate:	Butyrate:	BCFAs:
Production total SCFA				
Cellulose	49	15	22	14
FOS	52	21	21	6
F. serratus extract	48	24[a]	19	9
SCFA Production 0 – 24 h				
Cellulose	52	16	21	11
FOS	54	21	20	4
F. serratus extract	51	25[a]	18	6
SCFA Production 24 - 48 h				
Cellulose	25	12	28	34
FOS	34	16	31	30
F. serratus extract	20	13	33	33

(b)

	Acetate:	Propionate:	Butyrate:
Production total SCFA			
Cellulose	57	18	26
FOS	55	23	23
F. serratus extract	53	26[a]	21
SCFA Production 0 – 24 h			
Cellulose	58	17	24
FOS	56	23	21
F. serratus extract	55	27[a]	19
SCFA Production 24 - 48 h			
Cellulose	39	18	43
FOS	42	20	38
F. serratus extract	26	17	57

Table 3.2 To generate 16s rRNA bacterial gene amplicons (V4), a different version of the same forward primer was used for each fermentation sample. (a) Each version contained a unique a distinct multiple identifier (MID) barcode allowing for distinction between the different samples. (b) A mixture of four different reverse primers in conjunction with a single forward primer was used to generate16s rRNA bacterial gene amplicons.

(a)

Sample ID	Primer name	Clamp	Barcode	Oligo
R1 AT0	Fusion45bc1L	CCATCTCATCCC	AGAGAGAG	AYTGGGYDTAA
R1 BT0	Fusion45bc2L	CCATCTCATCCC	AGAGATGC	AYTGGGYDTAA
R1 CT0	Fusion45bc3L	CCATCTCATCCC	AGAGCAGC	AYTGGGYDTAA
R1 AT24	Fusion45bc9L	CCATCTCATCCC	AGATGCAG	AYTGGGYDTAA
R1 BT24	Fusion45bc10L	CCATCTCATCCC	AGATGCTC	AYTGGGYDTAA
R1 CT24	Fusion45bc11L	CCATCTCATCCC	AGCAGAGC	AYTGGGYDTAA
R3 AT0	Fusion45bc58L	CCATCTCATCCC	CTCAGATG	AYTGGGYDTAA
R3 BT0	Fusion45bc30L	CCATCTCATCCC	ATCTGCTC	AYTGGGYDTAA
R3 CT0	Fusion45bc31L	CCATCTCATCCC	ATGAGAGC	AYTGGGYDTAA
R3 AT24	Fusion45bc35L	CCATCTCATCCC	ATGATCTG	AYTGGGYDTAA
R3 BT24	Fusion45bc36L	CCATCTCATCCC	ATGATGAG	AYTGGGYDTAA
R3 CT24	Fusion45bc37L	CCATCTCATCCC	ATGCAGAG	AYTGGGYDTAA
R4 AT0	Fusion45bc41L	CCATCTCATCCC	CAGAGAGC	AYTGGGYDTAA
R4 BT0	Fusion45bc60L	CCATCTCATCCC	CTCAGCTC	AYTGGGYDTAA
R4 CT0	Fusion45bc43L	CCATCTCATCCC	CAGAGCAG	AYTGGGYDTAA
R4 AT24	Fusion45bc49L	CCATCTCATCCC	CAGCTCAG	AYTGGGYDTAA
R4 BT24	Fusion45bc50L	CCATCTCATCCC	CAGCTCTC	AYTGGGYDTAA
R4 CT24	Fusion45bc51L	CCATCTCATCCC	CATCTCTG	AYTGGGYDTAA

(b)

Primer	Clamp	Barcode	Oligo
Reverse01.1	CCTATCCCCTGTGTGCCTTGGCAGTCT	none	TACNVGGGTATCTAA
Reverse01.2	CCTATCCCCTGTGTGCCTTGGCAGTCT	none	CTACDSRGGTMTCTA
Reverse01.3	CCTATCCCCTGTGTGCCTTGGCAGTCT	none	TACCAGAGTATCTAA
Reverse01.4	CCTATCCCCTGTGTGCCTTGGCAGTCT	none	TACCRGGGTHTCTAA

Table 3.3 Rarefaction curve for each group at 97% similarities levels. The amount of taxonomical units (OTU's found as a function of the number of sequence tags sampled

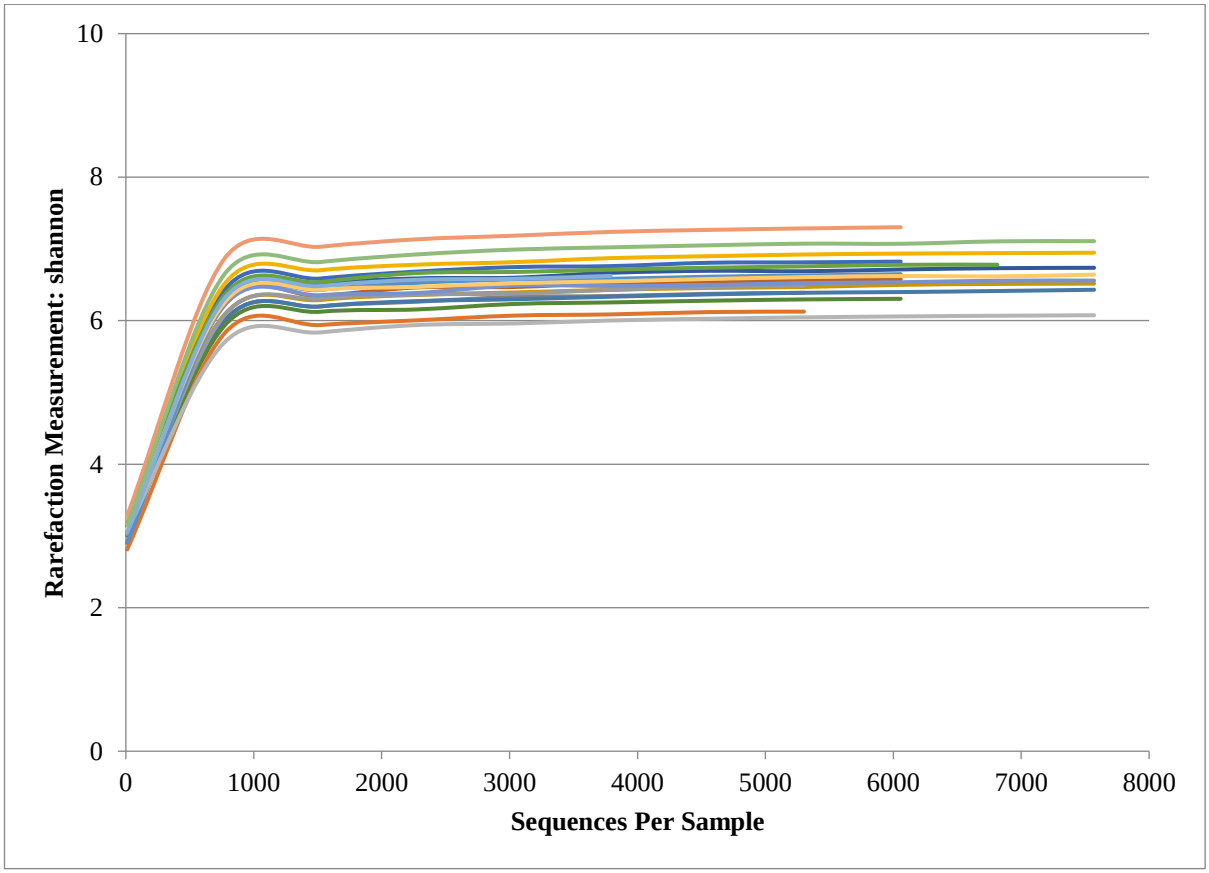


Figure 3.10 Alpha diversity was measured by several different metrics. (a) Shannon's index of diversity, observed species, Chao1 richness estimation, Simpson index of diversity, and phylogenetic diversity metrics were used to estimate alpha diversity

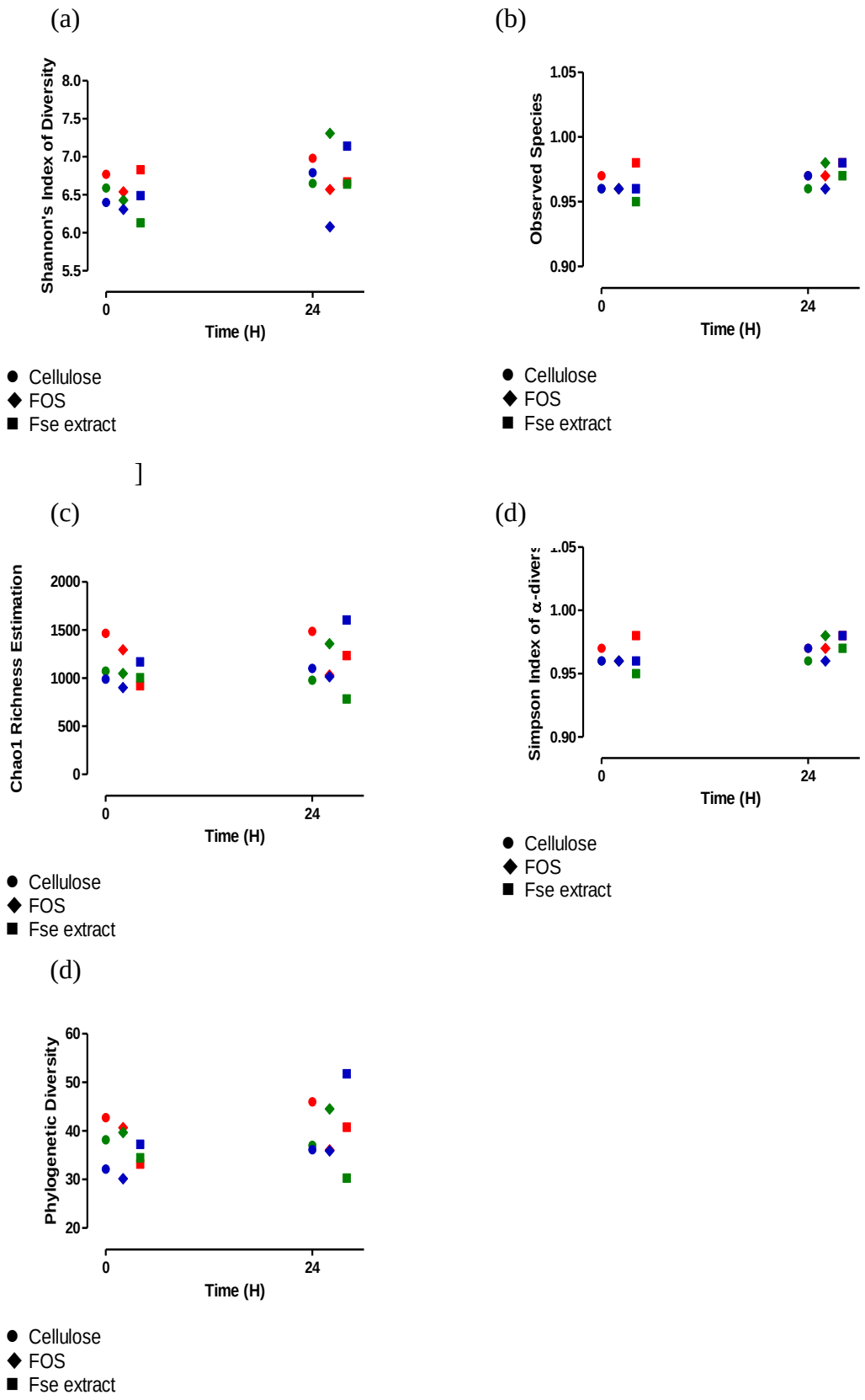


Figure 3.11 Beta diversity. Principle coordinate analysis of unweighted Unifrac reveals separation by fermentation run. Purple (light) - Run 1 0 h, Yellow (light) - Run 2 0 h, Green (light) - R3 0 h, Purple (dark) - Run 1 24 h, Yellow (dark) - Run 2 24 h, Green (dark) - R3 24 h.

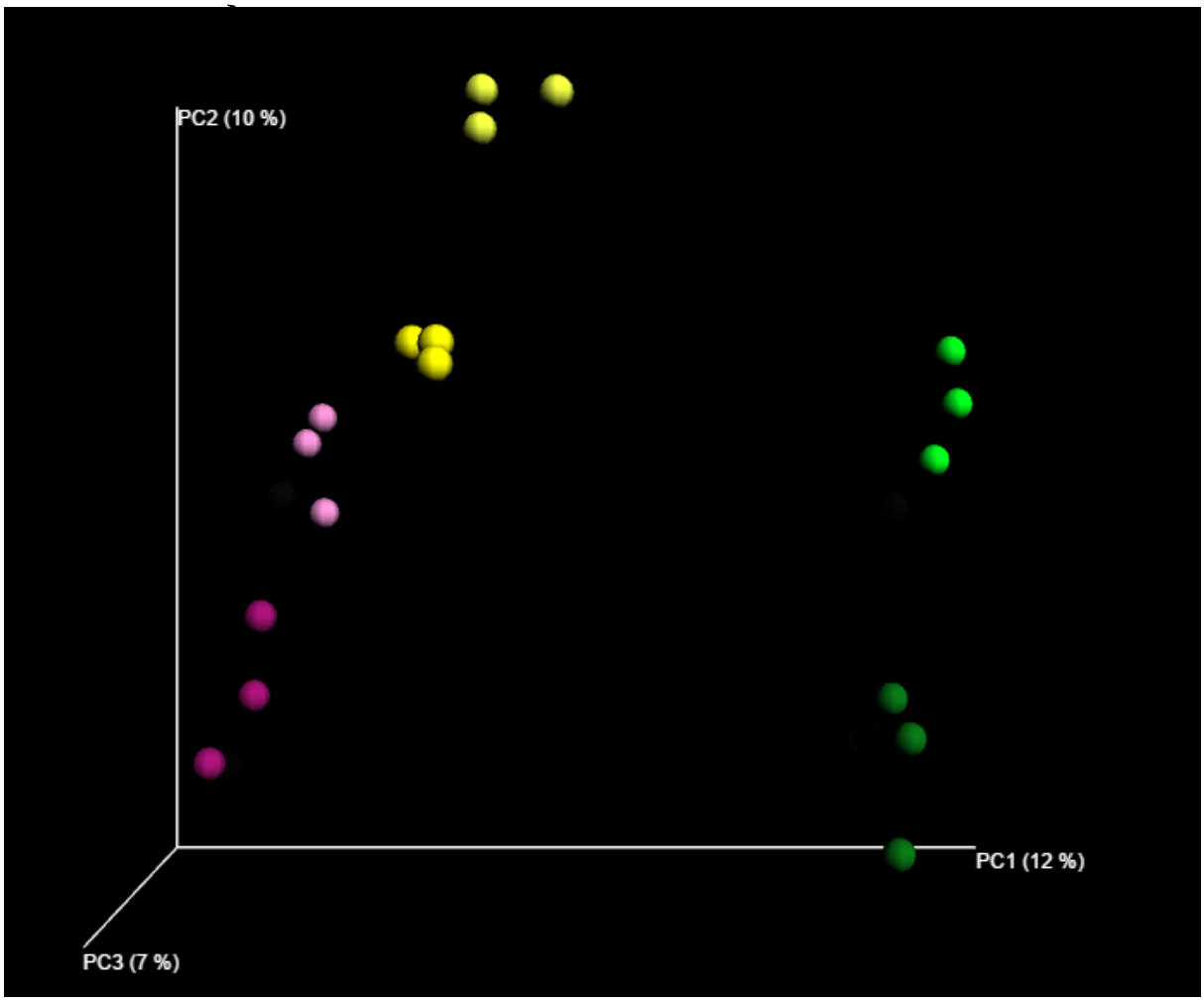


Figure 3.12 Phylum level comparisons of fermentation sample by overall abundance.

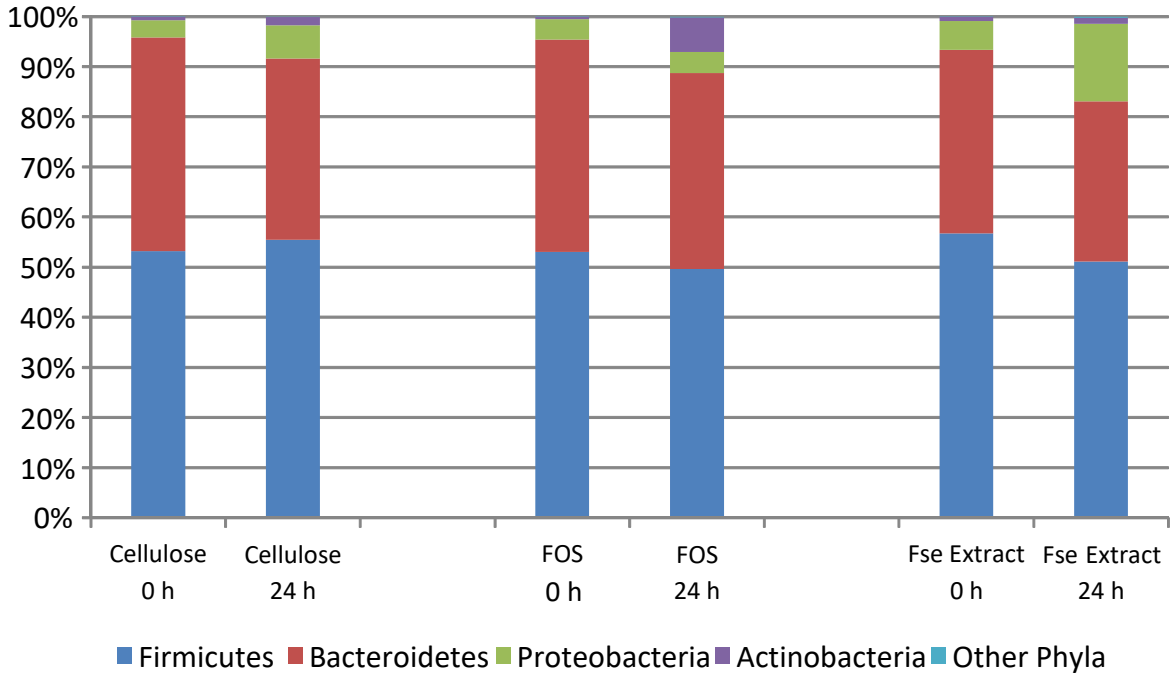
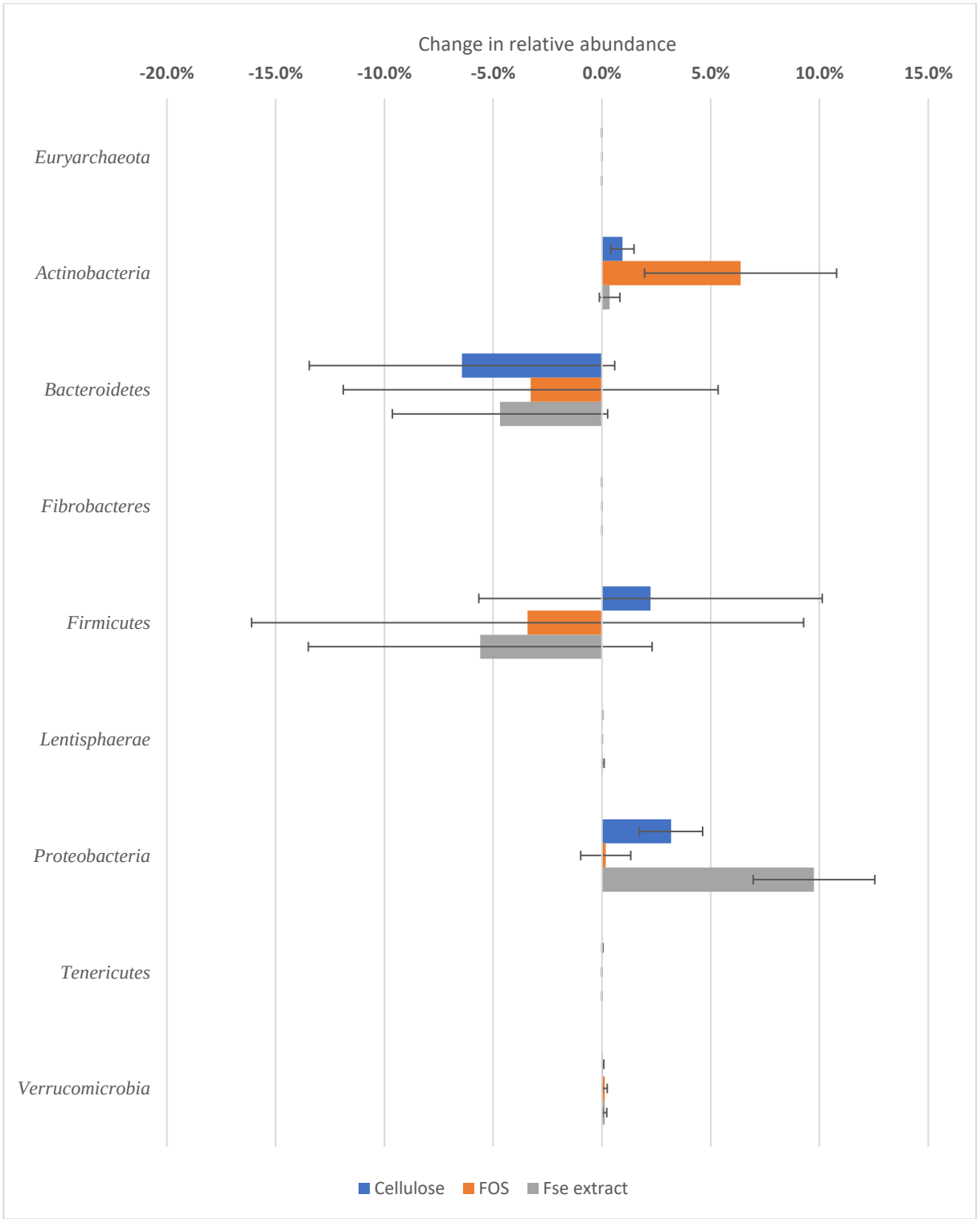


Figure 3.13 (a) Increase/decrease in relative abundance at the phylum level. (b) Percentage change in relative abundance at the phylum level. Data represent the mean (±SE).

(a)



(b)

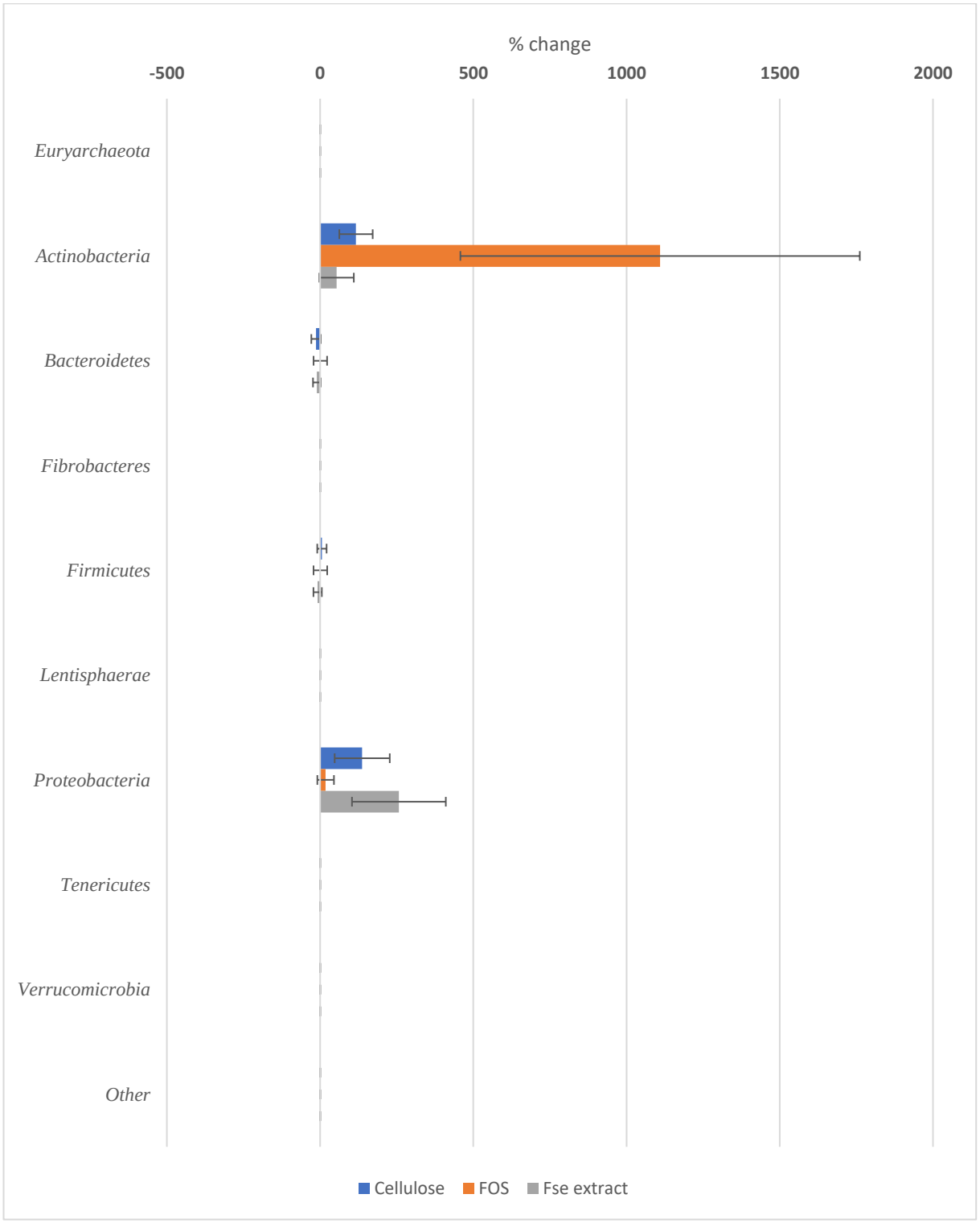
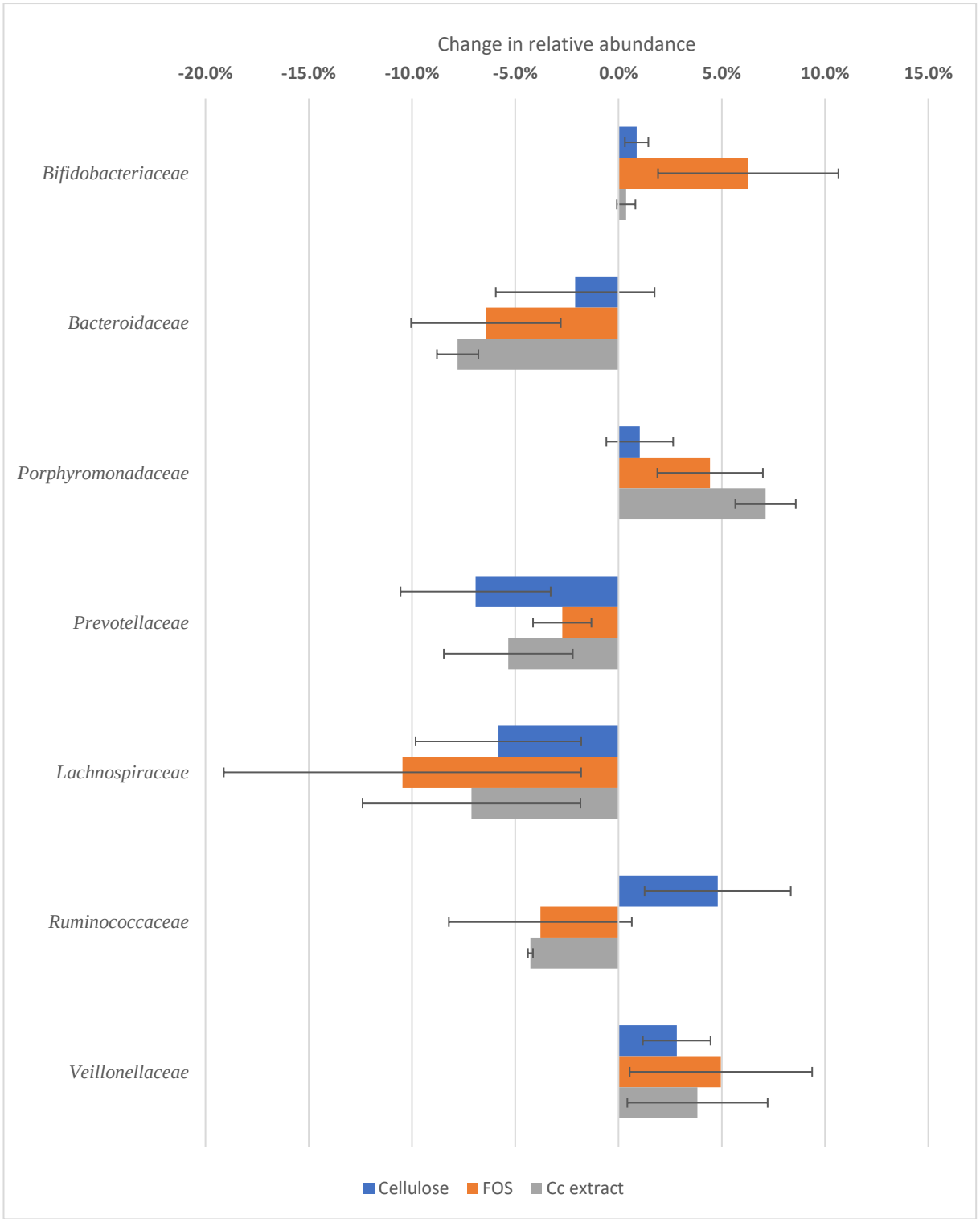
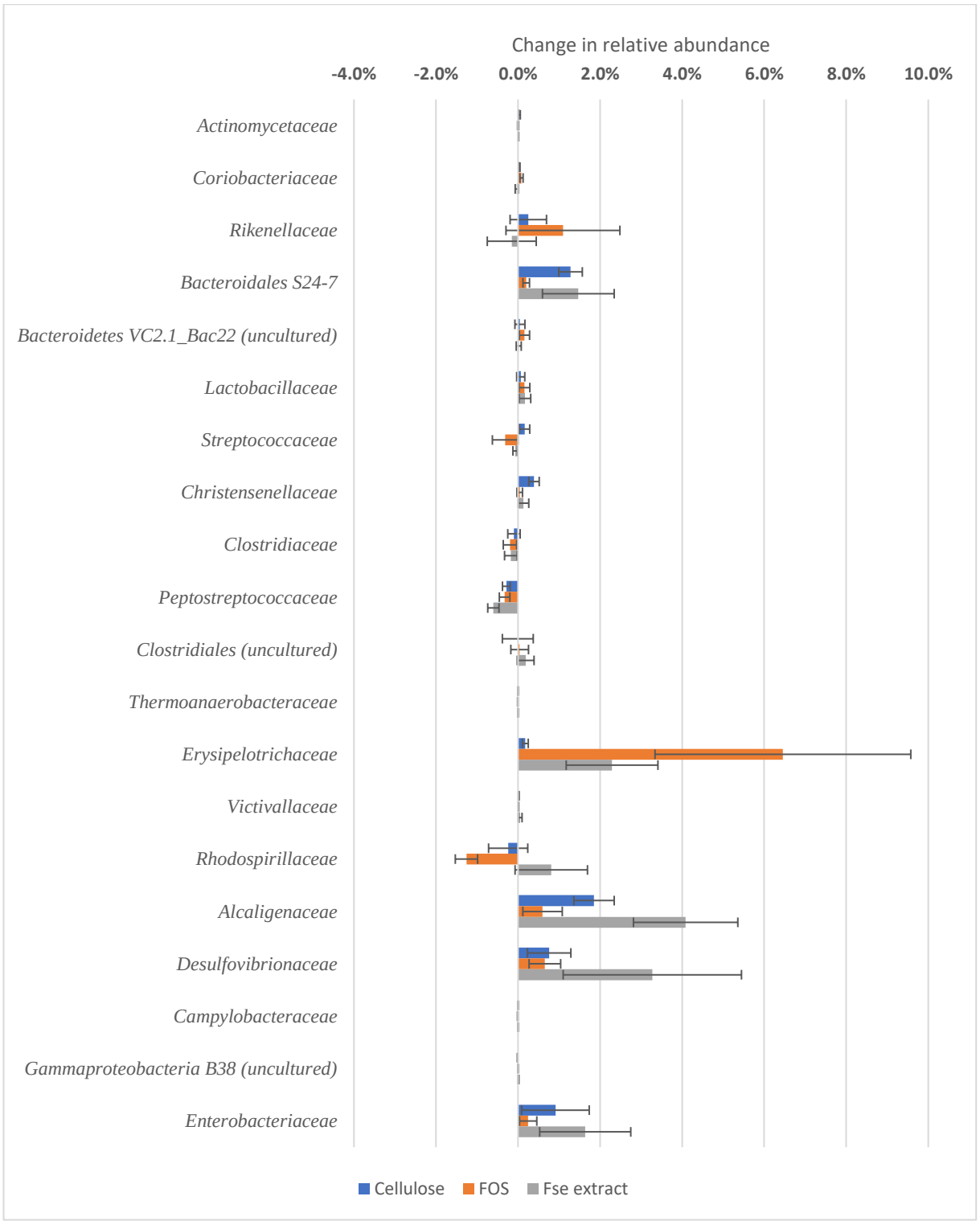

% change
-500
0
500
1000
1500
2000
Euryarchaeota
Actinobacteria
Bacteroidetes
Fibrobacteres
Firmicutes
Lentisphaerae
Proteobacteria
Tenericutes
Verrucomicrobia
Other
Cellulose
FOS
Fse extract

Figure 3.14. (a) Increase/decrease in relative abundance at the family level . (b) Percentage changes in the relative abundance at the family level. Values represent the mean (±SE).

(a)




Change in relative abundance
-4.0%
-2.0%
0.0%
2.0%
4.0%
6.0%
8.0%
10.0%
Actinomycetaceae
Coriobacteriaceae
Rikenellaceae
Bacteroidales S24-7
Bacteroidetes VC2.1_Bac22 (uncultured)
Lactobacillaceae
Streptococcaceae
Christensenellaceae
Clostridiaceae
Peptostreptococcaceae
Clostridiales (uncultured)
Thermoanaerobacteraceae
Erysipelotrichaceae
Victivallaceae
Rhodospirillaceae
Alcaligenaceae
Desulfovibrionaceae
Campylobacteraceae
Gammaproteobacteria B38 (uncultured)
Enterobacteriaceae
Cellulose
FOS
Fse extract

(b)

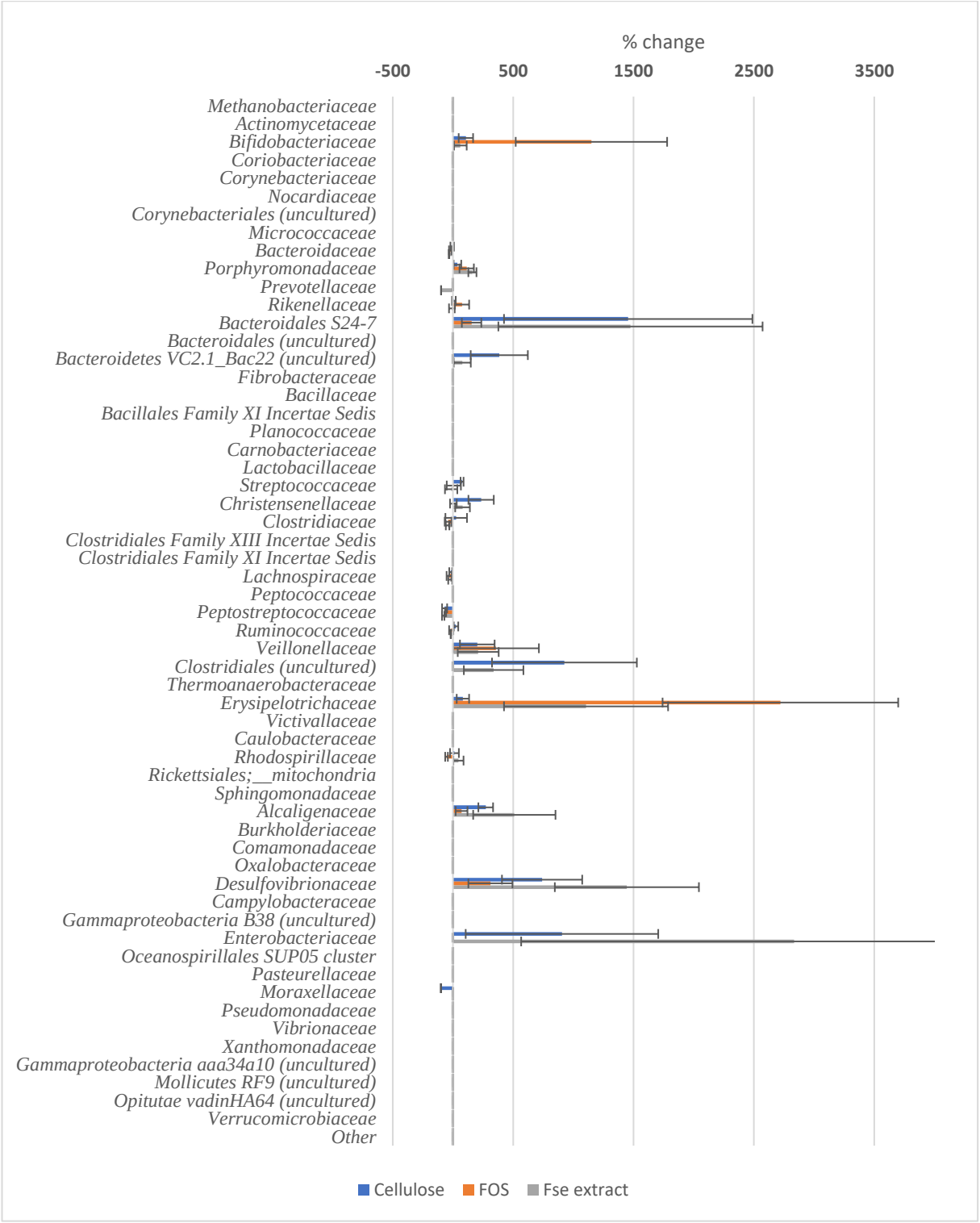
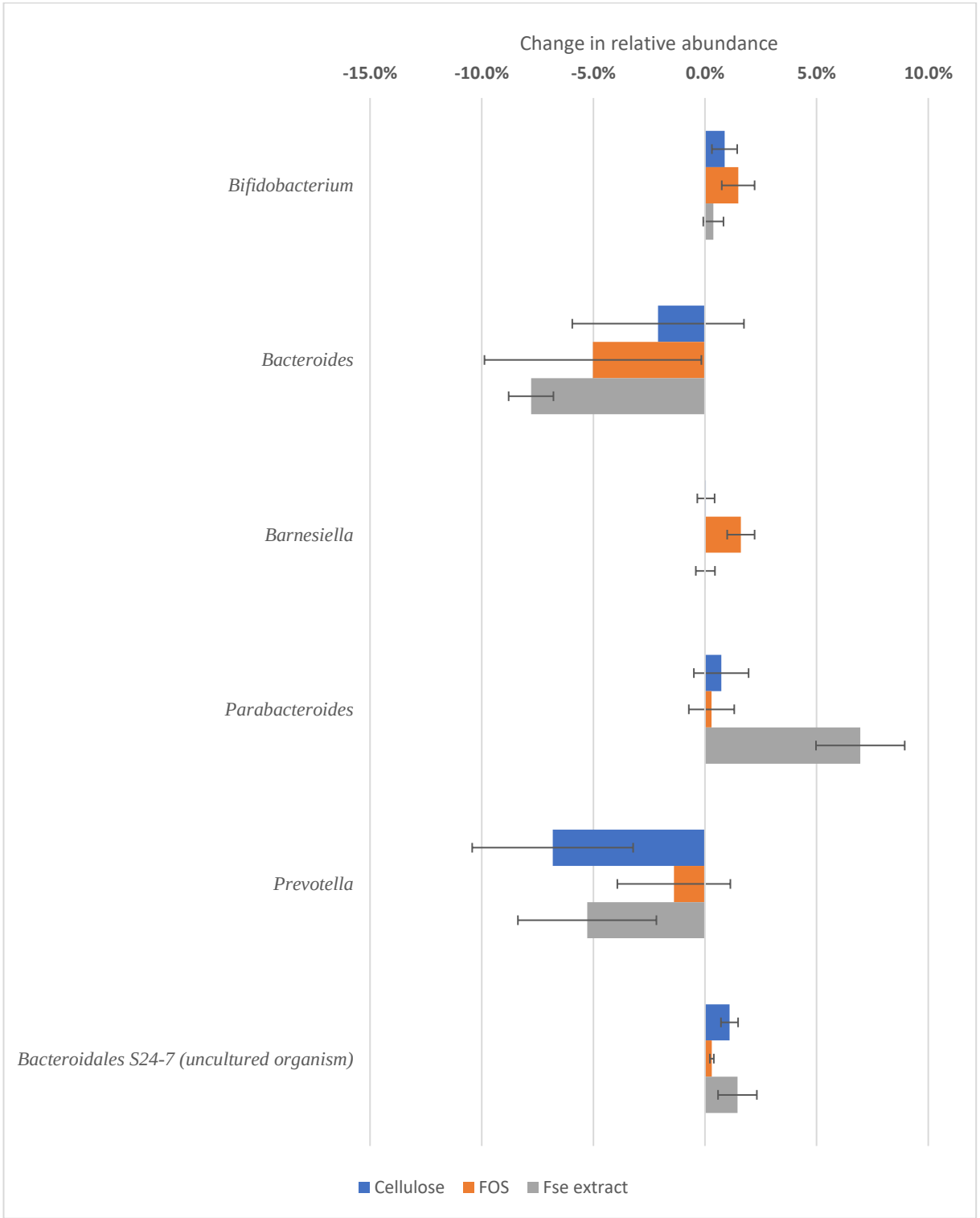
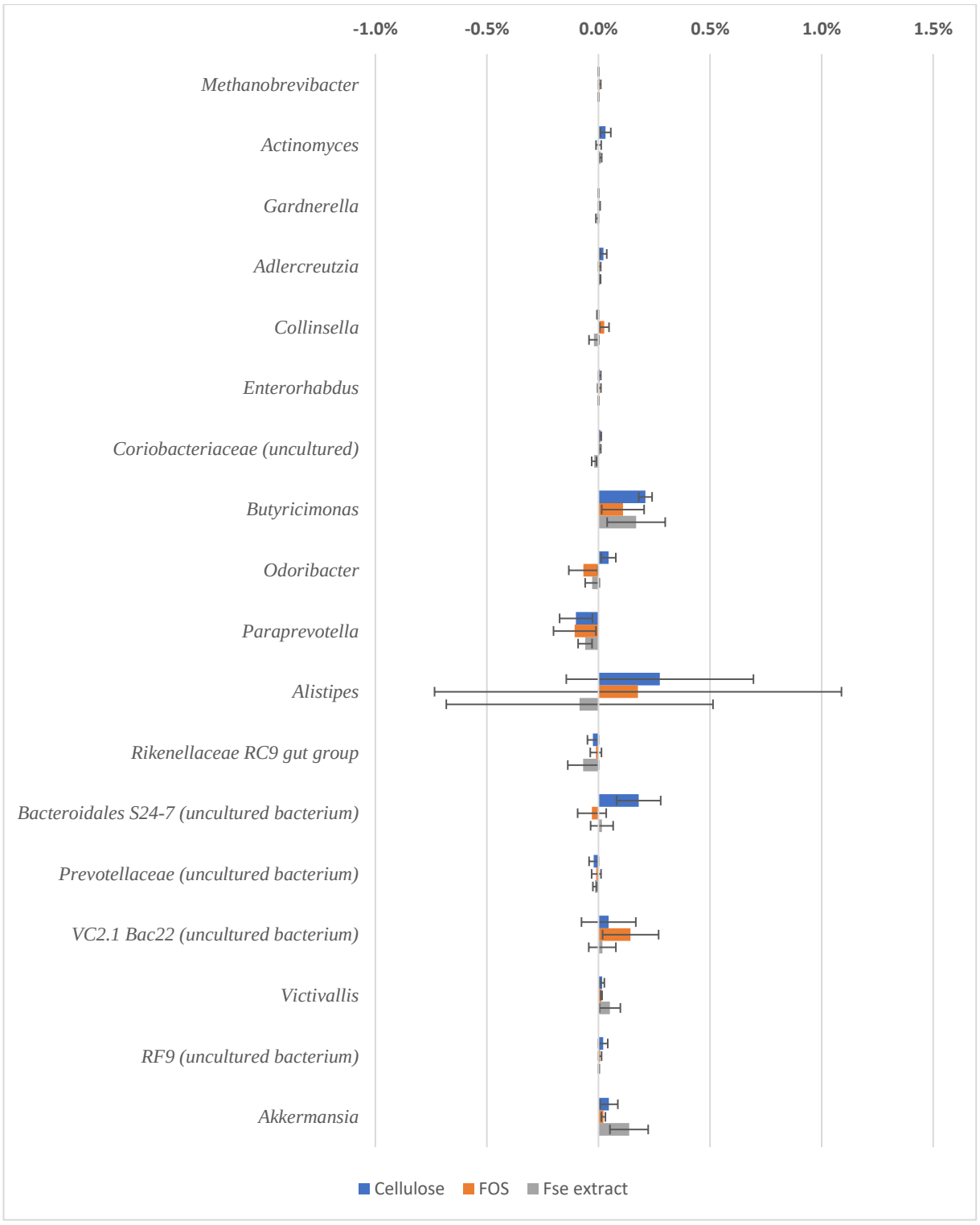

% change
-500
500
1500
2500
3500
Methanobacteriaceae
Actinomycetaceae
Bifidobacteriaceae
Coriobacteriaceae
Corynebacteriaceae
Nocardiaceae
Corynebacteriales (uncultured)
Micrococcaceae
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Bacteroidales S24-7
Bacteroidales (uncultured)
Bacteroidetes VC2.1_Bac22 (uncultured)
Fibrobacteraceae
Bacillaceae
Bacillales Family XI Incertae Sedis
Planococcaceae
Carnobacteriaceae
Lactobacillaceae
Streptococcaceae
Christensenellaceae
Clostridiaceae
Clostridiales Family XIII Incertae Sedis
Clostridiales Family XI Incertae Sedis
Lachnospiraceae
Peptococcaceae
Peptostreptococcaceae
Ruminococcaceae
Veillonellaceae
Clostridiales (uncultured)
Thermoanaerobacteraceae
Erysipelotrichaceae
Victivallaceae
Caulobacteraceae
Rhodospirillaceae
Rickettsiales;__mitochondria
Sphingomonadaceae
Alcaligenaceae
Burkholderiaceae
Comamonadaceae
Oxalobacteraceae
Desulfovibrionaceae
Campylobacteraceae
Gammaproteobacteria B38 (uncultured)
Enterobacteriaceae
Oceanospirillales SUP05 cluster
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Vibrionaceae
Xanthomonadaceae
Gammaproteobacteria aaa34a10 (uncultured)
Mollicutes RF9 (uncultured)
Opitutae vadinHA64 (uncultured)
Verrucomicrobiaceae
Other
Cellulose
FOS
Fse extract

Figure 3.15 (a) Increase/decrease in relative abundance of genera in the phyla Actinobacteria, Fibrobacteres, Lentisphaerae, Tenericutes, and Verrocomicrobia. (b) Percentage change in the relative abundance of genera in the phyla Actinobacteria, Fibroacteres, Lentisphaerae, Proteobacteria, Tenericutes, and Verrocomicrobia. Data represent the mean (±SE).

(a)




-1.0%
-0.5%
0.0%
0.5%
1.0%
1.5%
Methanobrevibacter
Actinomyces
Gardnerella
Adlercreutzia
Collinsella
Enterorhabdus
Coriobacteriaceae (uncultured)
Butyricimonas
Odoribacter
Paraprevotella
Alistipes
Rikenellaceae RC9 gut group
Bacteroidales S24-7 (uncultured bacterium)
Prevotellaceae (uncultured bacterium)
VC2.1 Bac22 (uncultured bacterium)
Victivallis
RF9 (uncultured bacterium)
Akkermansia
Cellulose
FOS
Fse extract

(b)

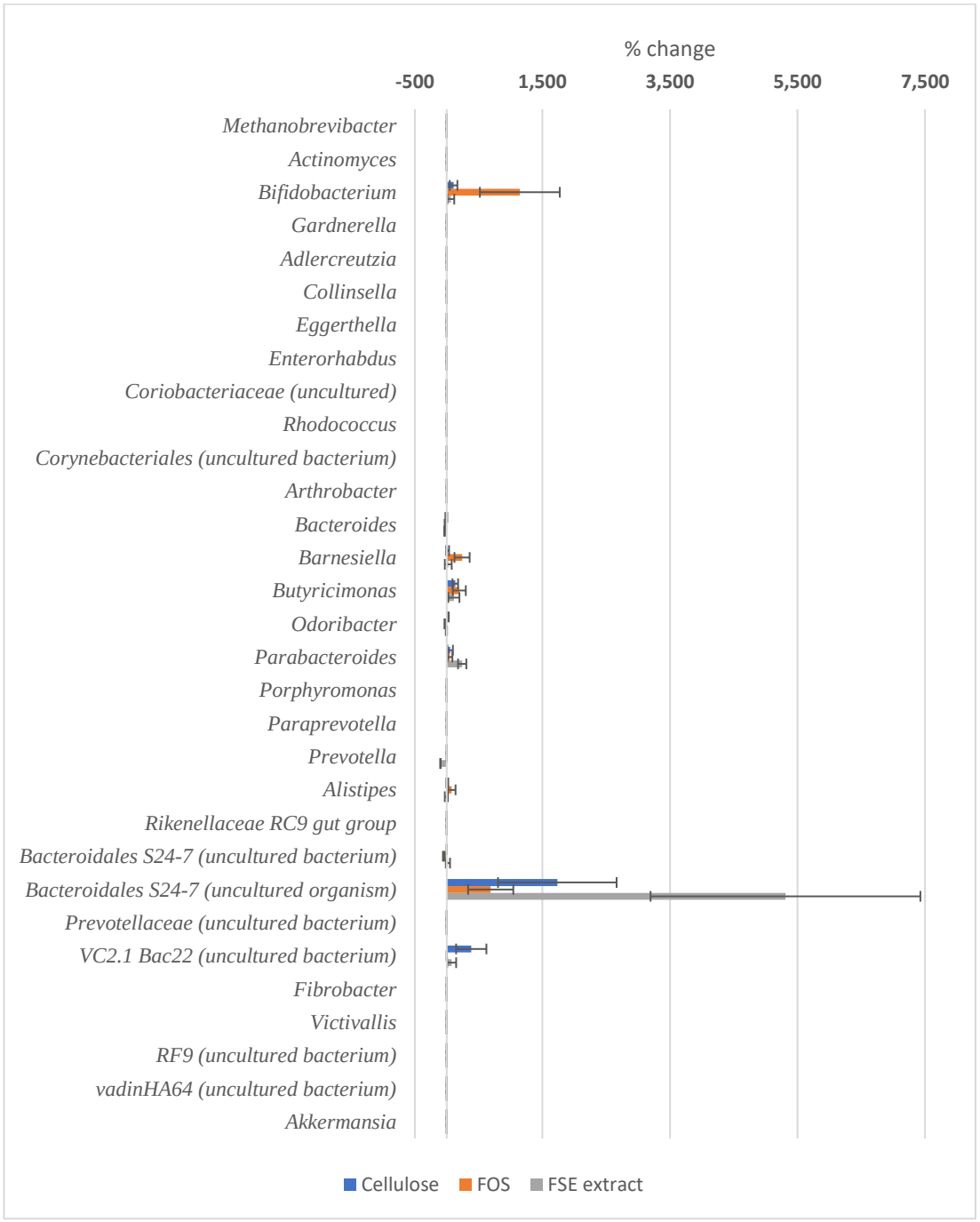
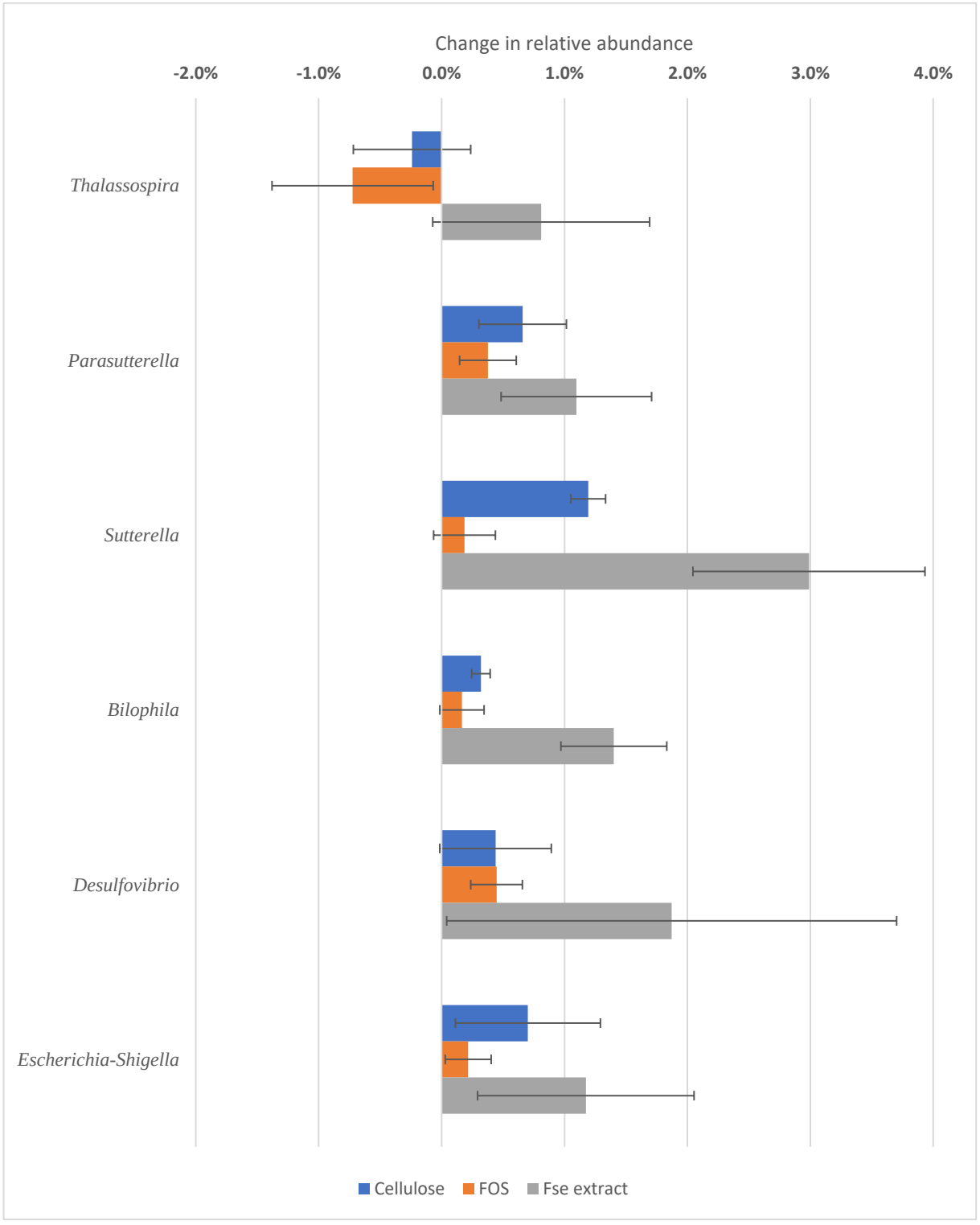
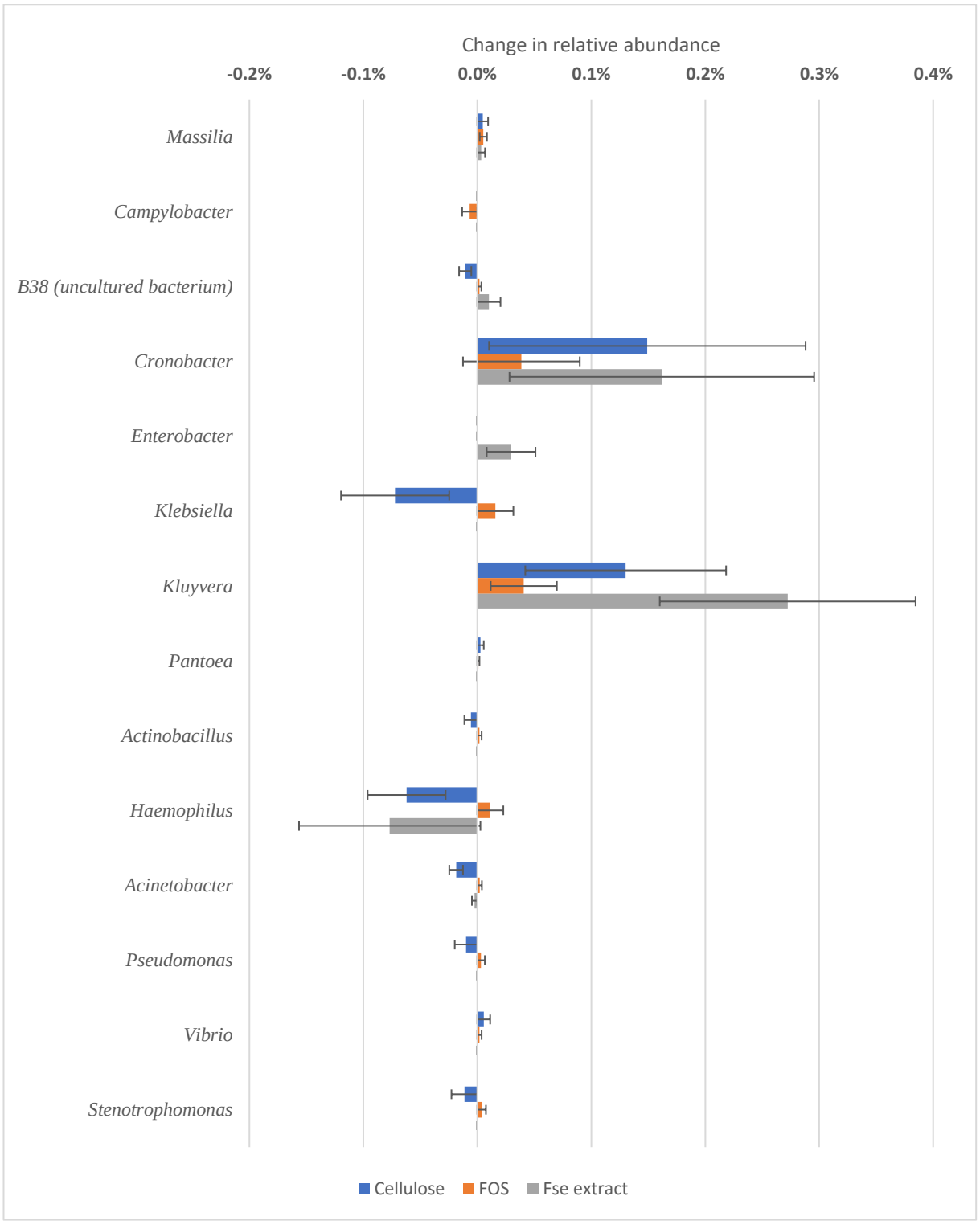

% change
-500
1,500
3,500
5,500
7,500
Methanobrevibacter
Actinomyces
Bifidobacterium
Gardnerella
Adlercreutzia
Collinsella
Eggerthella
Enterorhabdus
Coriobacteriaceae (uncultured)
Rhodococcus
Corynebacteriales (uncultured bacterium)
Arthrobacter
Bacteroides
Barnesiella
Butyricimonas
Odoribacter
Parabacteroides
Porphyromonas
Paraprevotella
Prevotella
Alistipes
Rikenellaceae RC9 gut group
Bacteroidales S24-7 (uncultured bacterium)
Bacteroidales S24-7 (uncultured organism)
Prevotellaceae (uncultured bacterium)
VC2.1 Bac22 (uncultured bacterium)
Fibrobacter
Victivallis
RF9 (uncultured bacterium)
vadinHA64 (uncultured bacterium)
Akkermansia
Cellulose
FOS
FSE extract

Figure 3.16. (a) Increase/decrease in relative abundance of the genera in the phylum Proteobacteria. (b) Percentage change in the relative abundance of genera in the phylum Proteobacteria. Values represent the mean (±SE).

(a)




Change in relative abundance
-0.2%
-0.1%
0.0%
0.1%
0.2%
0.3%
0.4%
Massilia
Campylobacter
B38 (uncultured bacterium)
Cronobacter
Enterobacter
Klebsiella
Kluyvera
Pantoea
Actinobacillus
Haemophilus
Acinetobacter
Pseudomonas
Vibrio
Stenotrophomonas
Cellulose
FOS
Fse extract

(b)

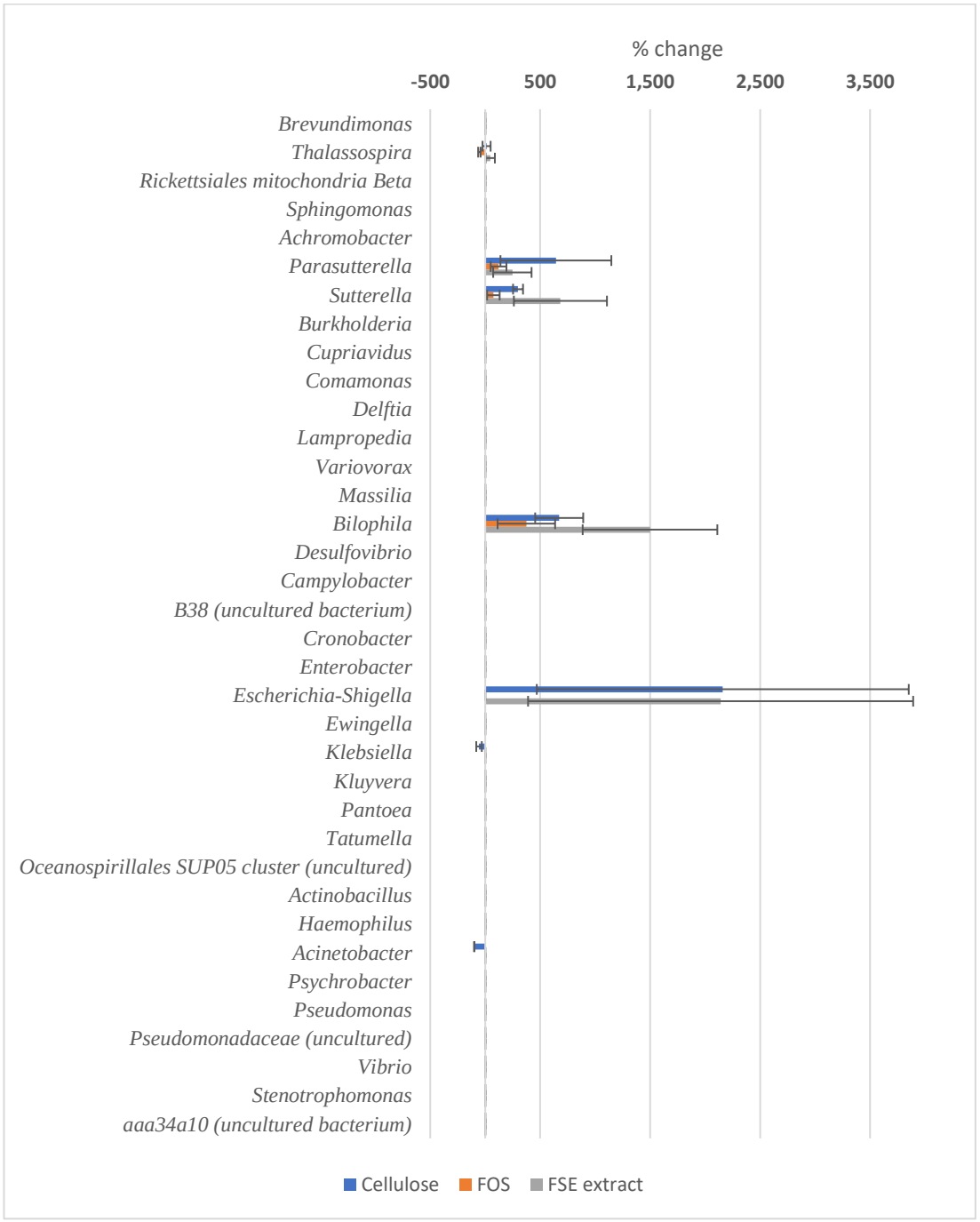
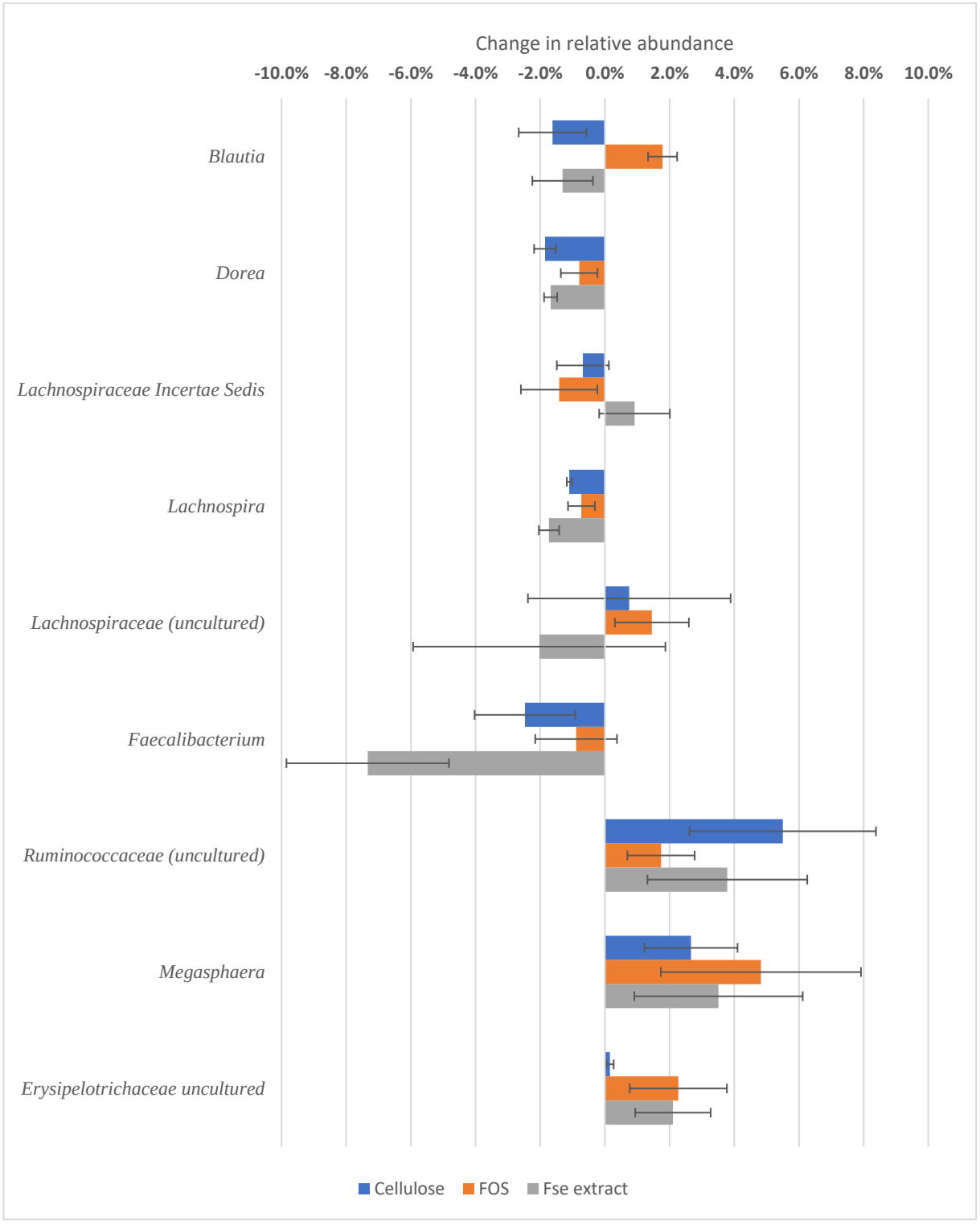
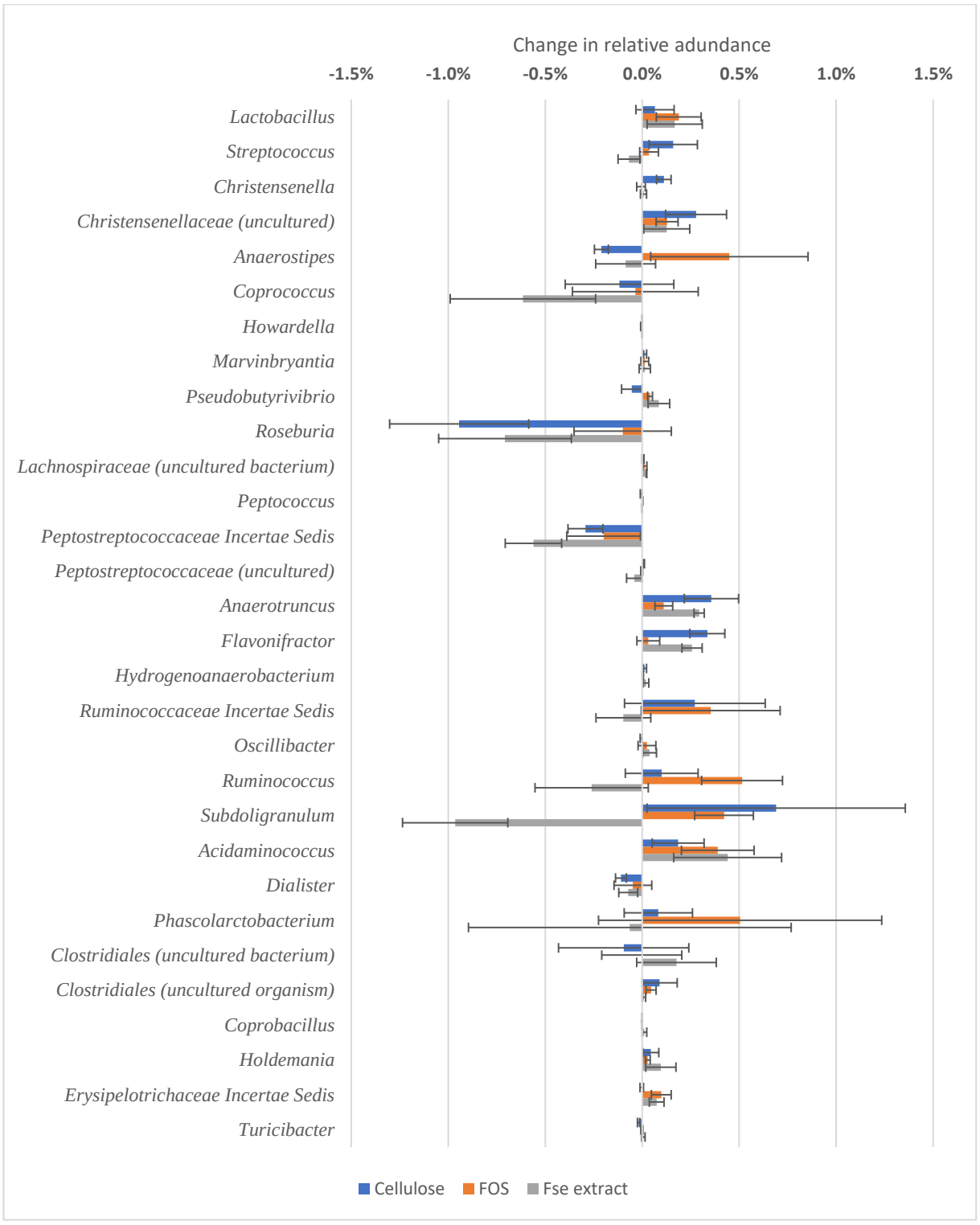

% change
-500
500
1,500
2,500
3,500
Brevundimonas
Thalassospira
Rickettsiales mitochondria Beta
Sphingomonas
Achromobacter
Parasutterella
Sutterella
Burkholderia
Cupriavidus
Comamonas
Delftia
Lampropedia
Variovorax
Massilia
Bilophila
Desulfovibrio
Campylobacter
B38 (uncultured bacterium)
Cronobacter
Enterobacter
Escherichia-Shigella
Ewingella
Klebsiella
Kluyvera
Pantoea
Tatumella
Oceanospirillales SUP05 cluster (uncultured)
Actinobacillus
Haemophilus
Acinetobacter
Psychrobacter
Pseudomonas
Pseudomonadaceae (uncultured)
Vibrio
Stenotrophomonas
aaa34a10 (uncultured bacterium)
Cellulose
FOS
FSE extract

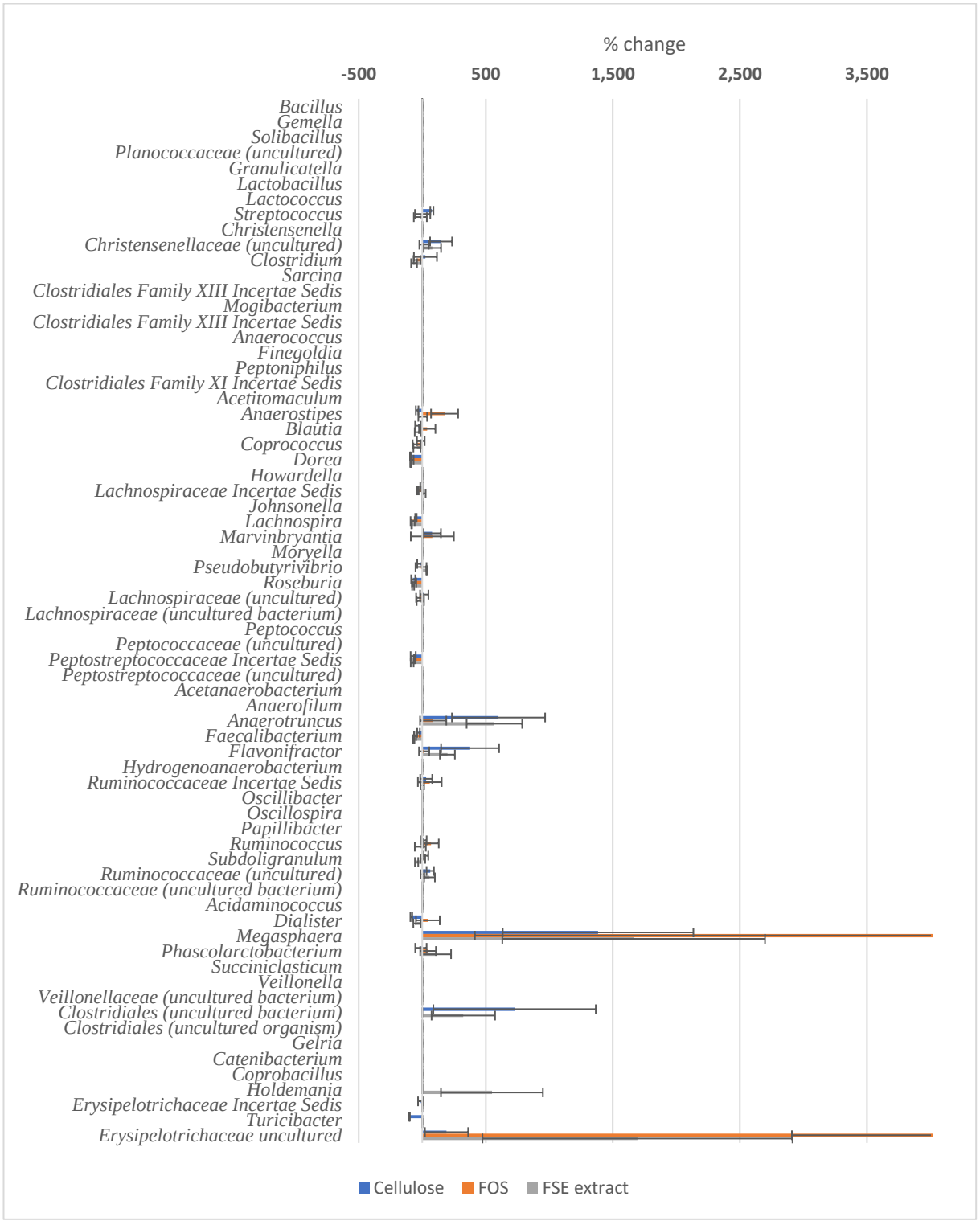
Figure 3.17 (a) Increase/decrease in relative abundance of genera in the phylum Firmicutes. (b) Percentage change in the relative abundance of genera in the phylum Firmicutes. Data represent the mean (±SE).

(a)

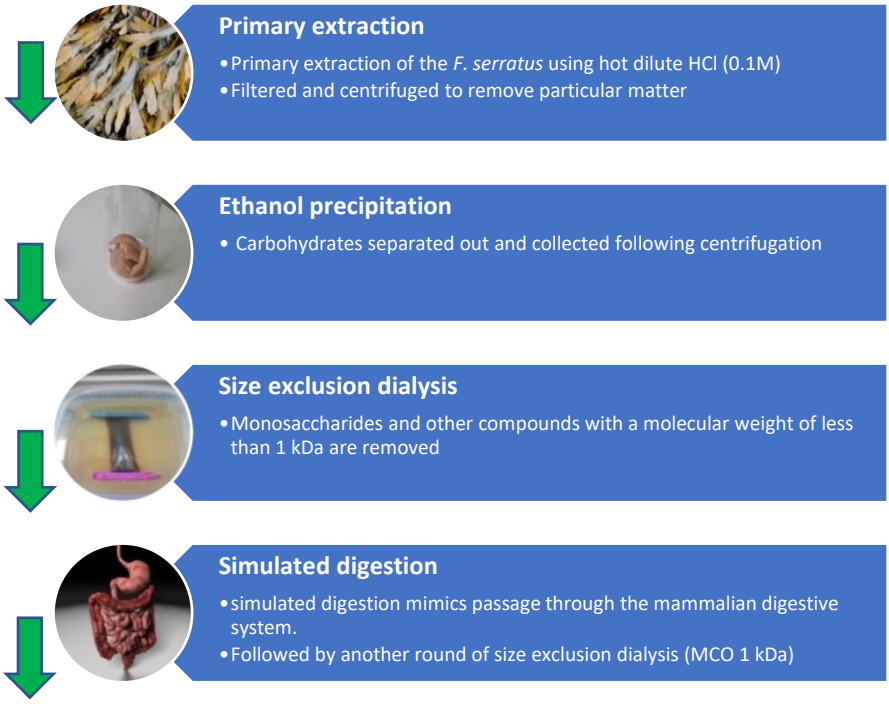



Change in relative adundance
-1.5%
-1.0%
-0.5%
0.0%
0.5%
1.0%
1.5%
Lactobacillus
Streptococcus
Christensenella
Christensenellaceae (uncultured)
Anaerostipes
Coprococcus
Howardella
Marvinbryantia
Pseudobutyrivibrio
Roseburia
Lachnospiraceae (uncultured bacterium)
Peptococcus
Peptostreptococcaceae Incertae Sedis
Peptostreptococcaceae (uncultured)
Anaerotruncus
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Ruminococcus
Subdoligranulum
Acidaminococcus
Dialister
Phascolarctobacterium
Clostridiales (uncultured bacterium)
Clostridiales (uncultured organism)
Coprobacillus
Holdemania
Erysipelotrichaceae Incertae Sedis
Turicibacter
Cellulose
FOS
Fse extract

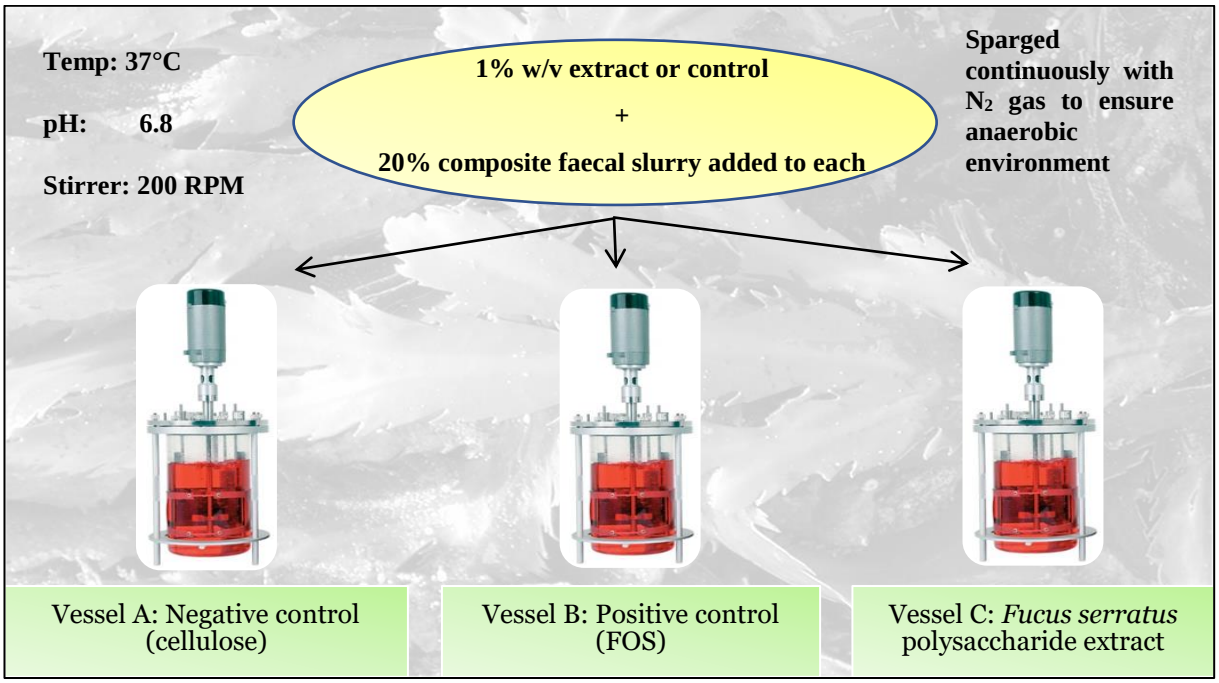
(b)


% change
-500
500
1,500
2,500
3,500
Bacillus
Gemella
Solibacillus
Planococcaceae (uncultured)
Granulicatella
Lactobacillus
Lactococcus
Streptococcus
Christensenella
Christensenellaceae (uncultured)
Clostridium
Sarcina
Clostridiales Family XIII Incertae Sedis
Mogibacterium
Clostridiales Family XIII Incertae Sedis
Anaerococcus
Finegoldia
Peptoniphilus
Clostridiales Family XI Incertae Sedis
Acetitomaculum
Anaerostipes
Blautia
Coprococcus
Dorea
Howardella
Lachnospiraceae Incertae Sedis
Johnsonella
Lachnospira
Marvinbryantia
Moryella
Pseudobutyrivibrio
Roseburia
Lachnospiraceae (uncultured)
Lachnospiraceae (uncultured bacterium)
Peptococcus
Peptococcaceae (uncultured)
Peptostreptococcaceae Incertae Sedis
Peptostreptococcaceae (uncultured)
Acetanaerobacterium
Anaerofilum
Anaerotruncus
Faecalibacterium
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Oscillospira
Papillibacter
Ruminococcus
Subdoligranulum
Ruminococcaceae (uncultured)
Ruminococcaceae (uncultured bacterium)
Acidaminococcus
Dialister
Megasphaera
Phascolarctobacterium
Succiniclasticum
Veillonella
Veillonellaceae (uncultured bacterium)
Clostridiales (uncultured bacterium)
Clostridiales (uncultured organism)
Gelria
Catenibacterium
Coprobacillus
Holdemania
Erysipelotrichaceae Incertae Sedis
Turicibacter
Erysipelotrichaceae uncultured
Cellulose
FOS
FSE extract

Supplementary Figure 3.1 Summary of the extraction process and post extraction processing carried out to produce a polysaccharide rich extract from *F. serratus*.



Supplementary Figure 3.2 Overview of the *ex vivo* faecal fermentation model used to assess the prebiotic potential of a polysaccharide rich extract from the brown seaweed *F. serratus.*



Supplementary Table 3.1. Alpha diversity., Shannon's index of diversity, observed species, Chao1 richness estimation, Simpson index of diversity, and phylogenetic diversity metrics were used to estimate alpha diversity.

Sample	Time	Chao1	Simpson	Shannon	Phyloge-	Observed
Control run 1	0 h	1,465.43	0.97	6.77	42.74	770.00
Control run 2	0 h	1,072.83	0.96	6.59	38.17	620.00
Control run 3	0 h	989.06	0.96	6.40	32.14	556.00
FOS run 1	0 h	1,295.62	0.96	6.54	40.69	688.00
FOS run 2	0 h	1,048.61	0.96	6.43	39.70	639.00
FOS run 3	0 h	903.48	0.96	6.31	30.18	505.00
Fse extract run 1	0 h	920.47	0.98	6.83	33.18	548.00
Fse extract run 2	0 h	1,004.17	0.95	6.13	34.47	522.00
Fse extract run 3	0 h	1,169.14	0.96	6.49	37.23	649.00
Control run 1	24 h	1,485.28	0.97	6.98	45.99	828.00
Control run 2	24 h	979.45	0.96	6.65	37.03	611.00
Control run 3	24 h	1,101.78	0.97	6.79	36.14	631.00
FOS run 1	24 h	1,031.50	0.97	6.57	36.09	619.00
FOS run 2	24 h	1,359.05	0.98	7.31	44.53	759.00
FOS run 3	24 h	1,014.92	0.96	6.08	35.91	561.00
Fse extract run 1	24 h	1,234.12	0.98	6.67	40.77	722.00
Fse extract run 2	24 h	784.16	0.97	6.64	30.28	485.00
Fse extract run 3	24 h	1,605.02	0.98	7.14	51.76	915.00

Chapter 4

A comparison of prebiotic properties between low molecular weight and high molecular weight polysaccharide extracts derived from Irish *Laminaria digitata.*

Kenneth Collins Chapter Contributions

Experimental:

Jointly performed all experiments relating to:

- The faecal fermentation using an *ex vivo* colonic model
- Analysis of SCFA production by GC-FID
- Extraction and purification of DNA from faecal pellets
- Generated amplicons for 454-sequencing

4.1 Abstract.

The proper functioning of dietary polysaccharides is greatly dependent on their molecular weight. These molecules are often chemically transformed into lower molecular oligosaccharides via depolymerisation processes. Lower molecular weight oligosaccharides can be better sources of carbon and energy for bacteria than their parent sugars. Seaweeds such as the brown seaweed *Laminaria digitata*, amongst others, are naturally rich in polysaccharides making them suitable candidates for prebiotic investigation. However, the fibre content of seaweeds is typically high molecular weight and while some demonstrate fermentative capacity in the lower intestines most pass through the gut too quickly for the gut microbiota to utilize them to any great extent. Molecular weight is an important factor for the correct functioning of polysaccharides. Lower weight oligosaccharides can be better sources of carbon and energy than their parent molecule. Here, two similarly processed extracts *L digitata* were used to access the effect of depolymerisation on prebiotic potential. Both extracts, the *L. digitata* polysaccharide extract (crude extract) and the *L. digitata* depolymerised polysaccharide extract (depolymerised extract), were subjected to an *ex vivo* faecal fermentation model where samples were taken at 0 h, 5 h, 10 h, 24 h, 36 h and 48 h for DNA sequencing, enumeration of *Bifidobacterium* and *Lactobacillus,* and SCFA analysis The fermentation of both extracts brought about a significant increase ($P<0.05$) in total SCFA production (1.8-fold and 1.7-fold increases) and the production of the biologically significant SCFAs, butyrate (1.7-fold and 0.9-fold increases), propionate (3.3-fold and 3.1-fold increases) and acetate (1.8-fold and 1.9-fold increases) in comparison with a cellulose control. It was found that depolymerisation of *L. digitata* polysaccharides significantly ($p<0.05$) increased propionate production and significantly ($P<0.05$) reduced butyrate production relative to the non-depolymerised *L. digitata* extract. Neither *L. digitata* extract had a stimulatory effect on *Bifidobacterium* or *Lactobacillus*.

4.2 Introduction.

The functionality of dietary polysaccharides is closely related to their molecular weight. Biopolymeric substrates, specifically polysaccharides, are often chemically transformed into lower molecular weight oligosaccharides or monosaccharides via depolymerisation processes, which can be hydrolytic, thermal or oxidative in nature. Depolymerised products can be better sources of carbon and energy for growing bacterial cells [1, 2]. There is emerging evidence that low-molecular weight polysaccharides and oligosaccharides derived from hydrocolloids can act as a source of soluble fibre and may also have prebiotic activity. Seaweeds such as *L. digitata*, amongst others, are naturally rich in polysaccharides making them suitable candidates for prebiotic investigation. However, the fibre content of seaweeds is typically high-molecular weight and, while some demonstrate a degree of fermentative capacity in the lower intestines, most pass through the gut too quickly for the microbiota to use them to any significant degree. The inclusion of fibre in the diet is known to increase the feeling of satiety following meals and improve digestive transit through increased faecal bulking [3]. Both *Bifidobacterium* and *Lactobacillus* have been observed to more selectively ferment lower molecular weight oligosaccharides than their parent carbohydrate with a higher molecular weight. An example of this is the degree of polymerisation (dp) of fructans, which has a major impact on their fermentation by probiotic bacteria, and thus has an influence on their beneficial effect on host health. Different dp of fructans can also influence the production profile of short chain fatty acids (SCFAs). For most probiotic strains, inulin-type fructans with a lower dp lead to earlier growth of bifidobacteria and lactobacilli than those with a higher dp. Longer chain inulins, however, show a more pronounced prebiotic effect affecting probiotic strains in the proximal and distal colon. The dp of gluco-oligosaccharides is also known to influence bifidobacterial selectivity, with a dp of 3 to 7 giving highest prebiotic activity. Gluco-oligosaccharides with a dp of greater than 7 were found to be selective for bifidobacteria in a non pH-controlled fermentation experiment [4, 5].

Fucoidans are high-molecular weight sulphated fucose rich polysaccharides which are extracted from brown seaweeds. Low-molecular weight fucoidan (< 30 kDa) can be obtained through acid hydrolysis or free radical depolymerisation. A study by Park et al., demonstrated that low-molecular weight fucoidan shows more potent

bioactivities than high-molecular weight fucoidan [6]. Low molecular weight fucoidan in combination with high stability fucoxanthin has also been found to exert a prebiotic effect and anti-inflammatory activity in Caco-2 cells by enhancing intestinal epithelial barrier and immune function [7]. In a study carried out by Tsai et al. low-molecular weight chitosan, a marine polysaccharide found in the shells of crustaceans, exhibited a significantly higher activity for promoting the growth of *Bifidobacterium* and mediating higher levels of total anaerobes than a fructooligosaccharide (FOS) control using a hamster model [8]. For the preparation of low molecular weight extracts, acid, radical and enzymatic methods have been widely described in literature. For the acid method, the higher temperatures or acidity can lead to lower molecular weight products as well as a lower sulphated group content. However, the sulphated group has been associated with many polysaccharide bioactivates. Enzymes prepared from bacteria and the digestive glands of marine invertebrates are highly specific for cleaving glycosidic bonds in the polysaccharide chain, though, the commercial preparation and usage of these enzymes is still not feasible. The radical method typically uses hydrogen peroxide. The presence of 0.1 – 10 mM hydrogen peroxide has been shown to hydrolyse polysaccharides such as xylan, galacturonan, arabinogalactan and cellulose [9].

Seaweeds and seaweed extracts have been shown to demonstrate prebiotic and immune modulatory activities. The approval of some seaweeds for human consumption has led, in part, to a renewal of interest in them as sources of dietary fibre and in their biological properties. In these seaweeds, soluble fibre consists of laminarin (β1–3, β1-6-glucan), fucans and alginates. The non-soluble fibre content is essentially cellulose. The human gastrointestinal tract (GIT) produces a limit array of enzymes that can catalyse the hydrolysis of various disaccharides and a few specific polysaccharides (starches). Most complex polysaccharides, such as FOS, however, cannot be degraded by human digestive enzymes [10, 11]. Laminarin is a glucose polymer consisting of a (1, 3), -β-D-glucan backbone with β (1, 6) branches while mannitol is a sugar alcohol. Alginate is a linear copolymer composed of (1, 4)-β-D-mannuronic acid and (1, 4)-α-L-guluronic acid. Laminarin and mannitol are storage carbohydrates that accumulate in the seaweed during the light season, while alginate is a structural component with little annual variation. The biomass composition of seaweeds depends greatly on many factors such as seaweed species, growing

conditions and the harvesting time [12]. Laminarin concentration varies with habitat and season, and can reach up to 32% of dry weight and is to be found mainly in the fronds of the different *Laminaria* species [13]. Laminarin contains two types of polymeric chains, the G-chain (glucose is attached to the end of the chain) and the M-chain (has mannitol as the terminal reducing chain). Fucoidan extracted from *L. digitata* is reported to contain fucose and sulphates as well as xylose, mannose, glucose, galactose and uronic acid in minor amounts [14]. *Laminaria digitata* is a brown seaweed generally found in the sub littoral zone of the northern Atlantic Ocean but can also be cultivated on ropes. The leaves of this species are known to grow up to 6m in length. Traditionally this seaweed has been used a fertilizer and for the extraction of iodine. In more modern times, *L. digitata* has been used as a source of alginic acid, which is used in the manufacture of toothpastes and cosmetics, and in the food industry as a binding, thickening and molding agent. The major structural component of the cell wall of brown seaweeds is alginate, which is composed of mannuronic and guluronic acids that are covalently linked in sequence together. The main storage carbohydrates of brown seaweeds are laminarin, which consists of a β-(1,3) glucan chain with small amounts of β-(1,6) branches, and mannitol. Other compounds of importance include fucoidan, proteins and minerals [15, 16].

Prebiotics are a class of food ingredients that are resistant to gastric acidity, hydrolysis by mammalian digestive enzymes and absorption in the GIT. They are fermentable in the gut and stimulate the growth and/or activity of intestinal bacteria associated with health and wellbeing. Carbohydrates such as FOS, inulin and galactooligosaccharides (GOS) are well accepted examples of prebiotics [17]. The most effective way to assess fermentation *in vitro* is through the incubation of a representative microbiota in an anaerobic fermentation chamber in the presence of potential prebiotic compounds [18]. Only a few studies have been carried out examining the impact that seaweed polysaccharides have on gut microbial communities, especially employing methodologies that allow for the analysis of most bacteria in the community rather than pre-determined groups [19]. The main aim of this study was to assess the effect of depolymerisation on the prebiotic potential of two similarly processed polysaccharide extracts, from the brown seaweed *Laminaria digitata* collected along the Irish coast using an *ex vivo* fermentation model. The two

extracts were termed the *L. digitata* polysaccharide extract (crude extract) and the depolymerised polysaccharide extract (depolymerised extract).

4.3 Materials and methods.

4.3.1 Materials.

The seaweed material used in this study to produce this polysaccharide rich extract was from the brown seaweed species *L. digitata*. The *L. digitata* raw material originated from a collection site in the Clare/Galway region of Ireland. All laboratory materials were obtained from Sigma-Aldrich, Dublin, Ireland unless otherwise stated. All plastic consumables were obtained from Sarstedt Ltd, Wexford, Ireland.

4.3.2 The primary extraction of *L. digitata* using a hot-acid extraction method.

The seaweed sample was washed with cold water to remove any attached particulate matter and afterwards stored at -20 °C. Prior to extraction, the seaweed was removed from cold storage and blended to a fine powder, which was then added to the reaction vessel and resuspended with deionised water (1:10 (w/v) seaweed/water solution). Hydrochloric acid (37%) was added (8.25 ml/L) to the seaweed/water solution to give a final concentration of 0.1 M. The vessel was allowed to shake at 75 rpm in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, Ireland) for 3 h at 70 °C. Afterwards, the vessel was removed from the shaker and allowed to cool. The seaweed solution was filtered through a muslin bag with the filtrate being transferred to a clean storage vessel. The remaining seaweed residue was returned to the reaction vessel with fresh reagents and a second extraction was performed under the same reaction conditions as above. Again, the contents were allowed to cool and were filtered once more, with the new filtrate being added to that which had previously been obtained. The combined filtrate was neutralised using NaOH (pH 6 - 8) and centrifuged at 5000 g for 5 min to remove remaining insoluble particulate matter prior to being blast-frozen and freeze-dried. The freeze-dried crude extract power was stored at - 20 °C prior to further refinement.

4.3.3 Ethanol precipitation of *L. digitata* extracts.

Algal sugars and polysaccharides were separated from the main seaweed bulk by ethanol precipitation. The freeze-dried crude extract powder was resuspended in minimal deionised water and reacted with ethanol (100%) using a ratio of seaweed: ethanol of 1:5. The seaweed ethanol mixture was then centrifuged for 5 min at 5000 g. The supernatant was discarded and the precipitate pellet containing the seaweed sugars was collected. Fresh deionised water was used to resuspend the pellet prior to being blast and freeze-dried

4.3.4 Size-exclusion dialysis and simulated digest of the *L. digitata* extract.

Simple sugars and other compounds were removed from the extract using size exclusion dialysis tubing with a molecular cut-off point of 1 kDa (Spectrum Labs, Breda, The Netherlands). The freeze-dried extract was resuspended in minimal deionised water with the 1 kDa dialysis tubing being cut into strips of approximately 15 cm in length. Each strip of tubing was rinsed gently with deionised water to remove traces of the sodium azide storage solution before use. The strips were filled with resuspended extract and sealed using clips. The tubing was positioned gently in a washed container filled with deionised water, covered with tin foil and placed in an orbital shaker at 25 °C at 40 rpm. The water in each container was replaced with fresh deionized water every day for three days. After the third day, the dialysis tubing was opened and the contents were collected. Following dialysis, a simulated digest as described by Connolly et al. [20] was performed with modifications. Briefly, α-amylase (200 U) was dissolved in filter sterilized $CaCl_2$ (1 mM, pH 7). This was added to the seaweed mixture and incubated while shaking (150 rpm) at 37 °C for 30 min. The pH was adjusted to pH 2 using HCl. Pepsin (2.7 g) was prepared in 125 ml 0.1 M HCl and added to the seaweed mixture which was then incubated under the same conditions as before for 2h. The pH was adjusted to pH 7 with NaOH. Bile (3.5 g) and pancreatin (560 mg) prepared in 125 ml 0.5M $NaHCO_3$ were added. The mixture was set shaking (150 rpm) for a further 3h. Subsequently, the seaweed digest underwent a second 1 kDa size-exclusion dialysis step to remove breakdown components from the simulated digest to yield the final product.

4.3.5 Depolymerisation of *L. digitata* polysaccharides using hydrogen peroxide.

A low molecular weight depolymerised *L. digitata* extract was prepared as described by Ramnani et al., with modification [21]. Following the ethanol precipitation step, *L. digitata* extract was dissolved in 0.04% $FeSO_4$ (1:5 w/v) solution to which hydrogen peroxide (30% puriss grade) was added (1:40 v/v) using a ratio of extract to hydrogen peroxide of 1:25. The mixture was allowed to shake at 150 rpm in a water bath at 80 °C for 15 min. Following the depolymerisation reaction, the extract was blast-frozen and freeze-dried prior to storage. The effect of depolymerisation on molecular weight was not determined.

4.3.6 Preparation of the cellulose control.

The cellulose control for the study was processed in a similar manner to the final crude extract. Firstly, the cellulose was subjected to a simulated gastric digestion followed by size exclusion dialysis with a molecular cut off point of 1 kDa, as previously outlined. Secondly, the cellulose (post dialysis) was blast frozen and freeze-dried.

4.3.7 *Ex vivo* faecal fermentation distal colon model.

The medium used for the faecal fermentations was prepared according to Fooks et al [22]. The Fooks media consisted of: tryptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), KH_2PO_4 (0.04 g/l), K_2HPO_4 (0.04 g/l), $CaCl_2.6H_2O$ (0.04 g/l), $MgSO_4.7H_2O$ (0.01 g/l), sodium bicarbonate (2 g/l), tween 80 (2 ml/l), hemin (0.05 g/l), vitamin K1 (10 µl/l), cysteine HCl (1 g/l) and bile salts (0.5 g/l). The medium (800 ml) was pH adjusted to 6.8 and autoclaved at 121 °C for 15 min. On the morning of the experiment, 2 g (1% w/v) of either control or seaweed extract (crude extract or depolymerised extract) were dissolved in 160 ml of Fooks medium and added aseptically to their respective vessels in the MultiFors fermentation system (Infors UK Ltd, Surrey, UK). The media was sparged with nitrogen gas for at least 120 min beforehand and throughout the experiment to ensure that an oxygen-free anaerobic environment was established in the system. A minimum of three freshly voided faecal samples were collected from volunteers on the morning of the fermentation. The donors were all

healthy adults (age 22 to 50 y), had no history of bowl problems and had not taken antimicrobial agents in the previous six months. The samples were combined to form a composite faecal sample by weighting out equal amounts from each stool sample into a sterile filter stomacher bag (Seward, VWR, Dublin, Ireland) and then adding an appropriate volume of maximum recovery diluent (Oxoid, Fisher Scientific, Dublin) containing 0.05% L-cysteine hydrochloride adjusted to pH 6.5 (which had been boiled after autoclaving and allowed to cool in the anaerobic cabinet (Whitley A85 anaerobic workstation, DW Scientific, Shipley, United Kingdom) to give a 20% composite faecal solution. The combined samples were placed in a stomacher and homogenized for 90 sec to create the composite slurry. Immediately after homogenization, 40 ml of the faecal slurry were added to the fermentation vessels at a final volume of 200 ml. Samples (1ml aliquots) were taken at 0 h, 5 h, 10 h, 24 h, 36 h and 48 h for total short-chain fatty acid analysis, pyrosequencing and hydrogen sulphide production analysis. Plate counts were carried out at 0 h, 5 h, 10 h and 24 h to enumerate the main probiotic genera, *Bifidobacterium* and *Lactobacillus.* The negative control for this experiment was cellulose and the positive control was fructooligosaccharide (FOS). The faecal fermentation was repeated three times (n = 3) with samples being taken at each time point in duplicate.

4.3.8 Analysis of short-chain fatty acid production.

Fermentation output was determined by measuring changes in SCFA concentration in collected supernatant fractions. The analysis was performed using a Varian CP-3800 GC system incorporating a Flame Ionisation Detector (FID). The system was fitted with a Zebron ZB-FFAP capillary column (30m length x 0.32 mm internal diameter x 0.32 µm film thickness; Phenomenex, Cheshire, UK). Helium was supplied as the carrier gas at an initial flow rate of 1.3 ml/min. The initial oven temperature was 100 °C, maintained for 30 sec, raised to 180 °C at 8 °C/min and held for 1 minute, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min. The temperatures of the detector and the injection port were set at 250 °C and 240 °C respectively. Samples were taken for total SCFA analysis at 0 h, 5 h, 10 h, 24 h, 36 h, and 48 h in triplicate. Each sample was centrifuged immediately at 15000 g for 15 min to remove bacteria and other solids with the supernatant being stored at - 80 °C. Prior

to processing, the SCFA samples were thawed on ice, centrifuged for a further 3 min at 15000 g and filtered sterilized (0.22 µm). Samples were then diluted 1:5 with deionised water and 1 mM 2-ethylbutyric acid, made up in formic acid, was added to each sample as an internal control. A calibration curve was created using 10.0 mM, 8.0 mM, 4.0 mM, 2.0 mM, 1.0 mM and 0.5 mM concentrations of a seven SCFA standard mix. The injected sample volume was 0.5 µl. Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Additional vials containing standards were included in each run to maintain calibration and a cleaning injection of 1.2% formic acid was used before each analysis. The SCFAs investigated in this study were acetate, propionate, butyric acid, isobutyric acid, valerate, isovalerate and hexanoate. The internal standard used was 2 - ethylbutyric acid.

4.3.9 Enumeration of *Bifidobacterium* and *Lactobacillus* bacterial populations by plate count method.

Numbers of bifidobacteria and lactobacilli were enumerated using agar plates counts. Samples from each fermentation vessel were taken at 0 h, 5 h, 10 h, and 24 h for bacterial plate counts. The media used for bifidobacteria enumeration was modified de Man, Rogosa and Sharpe (mMRS) agar plates supplemented with 0.05% (w/v) L-cysteine hydrochloride and 100 µg/ml mupirocin (Oxoid, Fisher Scientific, Dublin). The mupirocin was prepared by adding 200 mupirocin discs (200 µg/discs) to 10 ml of mMRS broth, which was then set gently shaking on an orbital shaker for 10 min and filter sterilized (0.45 µm) before being added to 400 ml of molten mMRS agar. The mMRS agar was allowed to cool to 48 °C before the addition of the mupirocin. *Lactobacillus* selective agar (LBS; Difco, Becton-Dickson ltd, Dublin, Ireland) plates with glacial acetic acid (1.32 ml/L) were prepared for *Lactobacillus* enumeration. Serial dilutions of faecal aliquots (10^{-1} to 10^{-7}) were carried out in maximum recovery diluent (Oxoid). Plates were incubated anaerobically at 37 °C for 3 - 5 days before counting.

4.3.10 Preparation of DNA for high-throughput pyrosequencing.

Total bacterial genomic DNA was extracted from 1ml of fermentation sample that was collected at time points 0 h and 24 h using the PowerFecal DNA Isolation Kit (MO BIO, San Diego, USA) per the manufacturers' instructions. Extracted DNA was stored at -20 °C following extraction. With a view to using high throughput DNA sequencing and microbiota compositional analysis, 16s rRNA bacterial gene amplicons (V4) were generated using universal 16S rRNA primers predicted to bind to 94.6% of all 16S rRNA genes [23, 24]. A forward primer (5'- AYTGGGYDTAAAGNG) and a combination of 4 reverse primers, R1 (5'- TACNVGGGTATCTAATCC), R2 (5'- CTACDSRGGTMTCTAATC), R3 (5'- TACCAGAGTATCTAATTC) and R4 (5'- TACCRGGGTHTCTAAT.CC) were utilised. Different versions of the same forward primer. each containing a distinct multiple identifier (MID, were used for each fermentation sample. All the primers used in this study were synthesised by Eurofins Genomics. PCRs were carried out using an Applied Biosystems® 2720 Thermo cycler. A hot start step of 95 °C for 10 min preceded all PCR runs. Each PCR reaction was performed under the following experimental conditions: heated lid 110 °C, 94 °C for 2 min followed by 36 cycles of 94 °C for 1 min, 52° C for 1 min and 72 °C for 1 min. This was followed by a final temperature step of 72 °C for 2 min and a holding step at 4 °C. PCRs had a final volume of 50 µl comprising 25 µl of BioMix Red (MSC, Ireland), 1 µl forward primer (0.15 µM), 1 µl reverse primer (0.15 µM) (mix of 4), 5 µl template DNA, and 18 µl sterile PCR water. All PCRs were carried out in triplicate. PCR products were analysed using agarose gel electrophoresis (1.5% in 1x TAE buffer). The DNA products were subsequently cleaned using Agencourt AMPure XP (Beckman Coulter, California, USA) and quantified using the Quant-iT™ PicoGreen ® dsDNA Assay Kit (Invitrogen, Massachusetts, USA). All samples were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, UK) per 454 protocols.

4.3.11 Analysis of sequencing data.

Raw sequences were quality-trimmed using the Qiime Suite of programmes [25]; any reads not meeting the quality criteria (a minimum quality score of 25 and a sequence

length <150bp for 16S amplicon reads) were discarded. Operational taxonomic units (OTUs) were aligned using PyNAST [26] and taxonomy assigned using BLAST [27] against the SILVA SSURef [28] database release 111. Alpha and beta diversity diversities were generated using Qiime. Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev. A phylogenetic tree was calculated using the FastTree [29] software and the resulting principal coordinate analysis was visualised within KiNG.

4.3.12 Statistical analysis.

All results are presented as mean value (± SE). Independent t-tests were used to measure significance ($p<0.05$). All statistical analysis was carried out using GraphPad Prism version 5.0 for Windows. Unpaired student t-tests were carried out on SCFA data and plate counts.

4.4 Results.

The differences in prebiotic potential between low molecular weight and high molecular weight polysaccharide extracts derived from Irish *Laminaria digitata* were assessed through the enumeration of culturable bifidobacteria and lactobacilli on appropriate media, the analysis of short-chain fatty acid production using a GC-FID system and the high through-put sequencing analysis of fermentation samples taken at 0 h and 24 h.

4.4.1 Effect of crude and depolymerised *L. digitata* extracts on culturable *Bifidobacterium* and *Lactobacillus.*

Neither *L. digitata* extract had a positive impact on the growth of culturable bifidobacteria during the first 24 h of the fermentation (Fig 4.1). Further, no noteworthy significant increases ($p<0.05$) in numbers of bifidobacteria were seen with either extract at any time-point. With the FOS fermentation, a non-significant increase in *Bifidobacterium* numbers was recorded at 5 h and a significant increase ($P<0.005$) was recorded at 10 h. A decrease in numbers was observed at 24 h. The highest recorded level of bifidobacteria in the FOS fermentation was at 5 h. Similarly, neither extract had a positive effect on culturable lactobacilli in the first 24 h. Decreases in the number of lactobacilli during both extract fermentations recorded decreases in *Lactobacillus* numbers (Fig 4.1). Non-significant increases of culturable lactobacilli were observed during the FOS fermentation at both 5 h and 10 h, with the highest level of recoverable lactobacilli being recorded at 10 h. Between 10 h and 24 h, a large decrease in *Lactobacillus* numbers was recorded. No significant differences ($p<0.05$) were observed between the initial level at 0 h of culturable bifidobacteria in each of the fermentations indicating that the starting faecal composite sample can be considered homogenous.

4.4.2 Effect of fermentation of *L. digitata* extracts on the production of short-chain fatty acids (SCFAs).

SCFA production was measured using a Varian CP-3800 GC system incorporating a Flame Ionisation Detector (FID). All values reported are minus the baseline values recorded at time point 0 h, and are the mean value (±SE). The highest levels of total SCFA production through microbial fermentation occurred in the FOS positive control vessels (112.0 ± 2.8 mm/ml), followed closely by the crude (109.9 ± 17.7 mm/ml) and the depolymerised (106.7 ± 16.5 mm/ml) extracts. Total SCFA production in the cellulose control vessels was determined to be 39.1 ± 2.1 mm/ml. (Fig. 4.2). Total SCFA concentration with the FOS fermentation was significantly ($p<0.05$) increased at all time-points (t = 0 h, 5 h, 10 h, 24 h, 36 h and 48 h). Total concentration of SCFAs for both extracts was significantly increased at 10 h, 24 h, 36 h, and 48 h. No significant difference in total SCFA production was found between the crude extract and the depolymerised *L. digitata* extract (Fig 4.2). SCFA production was significantly increased during the FOS fermentation ($p<0.05$) between 0-5 h, 5-10 h and 10-24 h, during the crude extract fermentation 5-10 h and 10-24 h and during the depolymerised extract fermentation between 5-10 h, 10-24 h and 24-36 h (Fig 4.2)

4.4.3 Effect of fermentation of *L. digitata* extracts on acetate, propionate and butyrate production.

For all fermentation conditions, the combined production of acetate, butyrate and propionate accounted for more than 85% of total SCFAs produced; Cellulose control (87.1% ± 2.2, FOS (94.6% ± 1.2), crude extract (91.4% ± 8.6) and depolymerised extract (93.9% ± 1.5). Acetate was the major SCFA produced under all fermentations conditions. Acetate production accounted for 51.4% ± 0.7 of total SCFA production with the cellulose control fermentation, 49.9% ± 4.0 with the FOS control fermentation, 49.8% ± 2.3 with the crude extract fermentation, and 55.1% ± 0.4 with the depolymerised extract respectively (Fig. 4.3). Total production of acetate was significantly increased ($P<0.05$) for the FOS fermentation (+ 177.5% ± 19.2), the crude extract (+ 176.1% ± 55.9), and the depolymerised extract (+ 191.0% ± 32.5), in comparison with cellulose (Fig. 4.4). There was no significant difference in total

acetate production observed between the crude and depolymerised extract. The concentration of acetate was significantly higher ($P<0.05$) than the cellulose control with the FOS fermentation at all time points (t = 5 h, 10 h, 24 h, 36 h and 48 h) and for both extract fermentations at 10 h, 24 h, 36 h and 48 h.

Acetate production was observed to be significantly increased for the FOS fermentation ($P<0.005$) between 0 – 5 h and 0 – 10 h, and ($P<0.05$) between 10 – 24 h, for the crude extract ($P<0.05$) between 5 – 10 h and 10 – 24 h and the depolymerised extract ($P<0.005$) between 5 – 10 h. There was no significant difference in total acetate production between the FOS control, the crude extract and depolymerised extracts. No significant difference was observed between acetate production for the crude extract and the depolymerised. at any timepoint (Fig. 4.4)

The second major SCFA produced during all fermentations was butyrate. This SCFA accounted for 21.8% ± 1.1 of total SCFAs production for the cellulose control, 27.5% ± 3.3 for the FOS fermentation, 22.0% ± 2.7 for the crude extract, and 15.4% ± 2.6 for the depolymerised fermentation (Fig. 4.3). Total production of butyrate was significantly higher ($p<0.005$) for the FOS fermentation (+ 259.8% ± 27.1), for the crude extract fermentation (+ 174.0% ± 8.8) and for the depolymerised extract fermentation (+ 87.3% ± 18.1), than the cellulose control. Butyrate concentration was significantly higher for the FOS fermentation at all time points (t = 5, 10, 24, 36, and 48 hours) and for both *L. digitata* extracts at 10 h, 24 h, 36 h and 48 h (Fig. 4.5). Significant increases of butyrate during the FOS fermentation occurred between ($p<0.05$) for all time intervals for the crude extract ($P<0.005$) between 0 – 5 h), ($P<0.05$) between 5 – 10 h and ($P<0.005$) between 10 – 24 h and for the depolymerised extract $P<0.05$) between 0 – 5 h, 0 – 10 h, 5 – 10 h (+ 204.0% ± 43.3) and 24 – 36 h. (Fig.4.5). Production of butyrate was significantly higher ($P<0.05$) with the crude extract than the depolymerised extract between 10 – 24 h, Overall production of butyrate was also significantly increased ($P<0.05$) with the crude extract over the depolymerised extract (+ 50.3% ± 20.6). There was no significant difference in overall butyrate production between the crude extract and the FOS control, however the FOS control did generate significantly higher levels ($P<0.05$) of butyrate than the depolymerised extract (+ 95.8% ± 24.0).

The third major SCFA produced was propionate. Production of propionate accounted for 13.9% ± 1.0 of all SCFAs produced during the cellulose fermentation, 17.2% ± 2.8 with the FOS fermentation, 19.6% ± 1.8 with the crude *L. digitata* extract, and finally 23.4% ± 1.6 for the depolymerised extract fermentation (Fig. 4.3). Total production of propionate was significantly increased ($P<0.05$) for FOS fermentation (+ 224.0% ± 55.3), for the crude extract, (+ 333.7 % ± 57.4), and for the depolymerised extract (+ 313.6% ± 23.8), in comparison with cellulose. Propionate levels were significantly higher ($P<.0.05$) than the cellulose control for FOS fermentation at 10 h, 24 h, 36 h, and 48 h, while propionate levels for both extract fermentations were significantly higher at all time points (t = 5 h, 10 h, 24 h, 36 h, and 48 h) (Fig 4.6). A significant increase in propionate production was observed for the FOS fermentation ($P<0.05$) between 5 – 10 h and 10 – 24 h, with the crude extract ($P<0.005$) between 0 – 5 h, ($P<0.05$) 5 – 10 h, 10 – 24 h and 24 – 36 h and for the depolymerised extract ($p<0.005$) between 0 – 5 h, and 5 – 10 h. Production of propionate was significantly ($P<0.05$) higher with the depolymerised extract than the crude extract between 0-5 h. Total production of propionate was observed to be significantly increased ($p<0.05$) for the depolymerised extract over the crude *L. digitata* extract (+ 33.3% ± 6.9), between 0 – 5 h.

4.4.4 Effect of fermentation of *L. digitata* extracts on branched chain fatty acid production.

Accumulated BCFA production accounted for 12.9% ± 2.2 of total SCFA production for the cellulose fermentation, 5.4 % ± 1.2 for the FOS fermentation, 8.6 % ± 1.3 for the crude extract and 6.075 % ±1.495 for the depolymerised extract (Fig. 4.3). Total production of BCFAs, in comparison with the cellulose control, was significantly ($P<0.05$) increased in the presence of the crude extract (Fig. 4.7). A significant increase in production occurred for this extract between 36-48 h. No significant differences were observed between the crude and depolymerised extracts. Furthermore, no significant difference in BCFA production was observed between the FOS control and either *L. digitata* extract.

4.4.5 High throughput DNA sequencing of the 16s rRNA gene variable V4 region.

Values are only given for bacterial phyla, family and genera that were present in all samples.

4.4.6 Sequencing reads at the phylum level.

The dominant phyla present were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. These four phyla accounted for 98.5% ± 0.505 of all assigned sequencing reads at 0 h. For the cellulose control, there was an average increase in the relative amount of Proteobacteria (+ 266.0% ± 64.7), Actinobacteria (+ 33.1% ± 8.6) and Bacteroidetes (+ 7.5% ± 36.0), and an average decrease in the relative abundance of Firmicutes (- 12.4% ± 8.0) during the fermentation (Fig.4.8), in comparison with the cellulose control.

The FOS fermentation revealed a significant reduction ($P<0.05$) in the relative abundance of Proteobacteria (Fig. 4.8), in comparison to cellulose. For the crude extract, there was a significant decrease ($P<0.05$) in relative abundance of Fibrobacteres in comparison with the cellulose control (Fig. 4.8). For the depolymerised extract, there was a significant reduction ($P<0.05$) in relative abundance of Actinobacteria in comparison with cellulose. No significant differences were observed between the crude extract and the depolymerised extract for any measurable bacterial phyla. In comparison with the FOS control, a significant increase ($P<0.05$) in the change of relative abundance of Proteobacteria was observed with the crude extract. There were no significant differences in relative abundance of any genera with the depolymerised extract in comparison with the FOS fermentation (Fig. 4.8).

4.4.7 Sequencing reads at the family level.

The most prevalent bacterial families present in all fermentation vessels were *Ruminococcaceae, Lachnospirace, Prevotellaceae, Bacteroidaceae*, and *Rikenellaceae*. For FOS, in comparison with the cellulose control, there were

significant positive influences (P<0.05) on the relative abundance of the Clostridiales Family XIII Incertae Sedis (Fig 4.9) and the *Lachnospiraceae* (Fig. 4.9) and a significant reduction of relative abundance for *Streptococcace* and the *Alcaligenaceae* (Fig. 4.9). Sequencing reads at the family level with the crude extract fermentations returned significant positive influences (P<0.05) for *Porphyromonadaceae*, *Lachnospiraceae*, Erysipelotrichaceae and Gamma Proteobacterium B38 (Fig. 4.9) and a significant reduction (p<0.05) in relative abundance for *Fibrobacteraceae* and the *Streptococcaceae* (Fig 4.9) in comparison with cellulose.

For the depolymerised extract fermentations, there were significant positive influence on relative abundance (P<0.05) *Lachnospiraceae* and *Erysipelotrichaceae* (Fig 4.9) and decreases for the *Bifidobacteriaceae* and the *Alcaligenaceae* (Fig. 4.9), in comparison with cellulose. A significant difference was recorded in the level of Gamma Proteobacterium B38 between the crude extract and the *L digitata* depolymerised extract. In comparison with the FOS fermentations, the crude extract significantly increased (P<0.05) the relative abundance of *Porphyromonadaceae*, *Bacteroidales* family S24-7, *Planococcaceae* and Gamma Proteobacterium B38 (Fig. 4.9) and significant reduction in the relative abundance of *Clostridiales* (uncultured), Clostridiales Family XIII Incertae Sedis, and *Xanthomonadaceae*. In comparison with the FOS fermentations, the depolymerised extract was associated with significant increases in relative abundance of *Porphyromonadaceae* and Bacteroidales families S24-7 (Fig. 4.9) and decreases for Clostridiales (uncultured) *Peptrostreptococcaceae* and Clostidales Family XIII Incertae Sedis (Fig. 4.9).

4.4.7 Sequencing reads at the genus level.

At the genus level, the most prevalent bacteria present were uncultured *Ruminococcaceae* (Firmicutes), *Bacteroides* (Bacteroidetes), *Subdoligranulum* (Firmicutes), uncultured *Lachnospiraceae* (Firmicutes), *Lachnospiraceae* Incertae Sedis (Firmicutes) and *Faecalibacterium* (Firmicutes). The FOS fermentation resulted in a significant increase (P<0.05) in the relative abundance of Clostidales Family XIII Incertae Sedis (Fig 4.11) and significant decreases in relative abundance of *Sutterella* (Fig. 4.10), *Cronobacter* (Fig. 4.10), *Enterobacter* (Fig. 4.10). *Christensenella* (Fig

4.11), *Flavonifractor* (Fig. 4.11), *Butyricimonas* (Fig 4.12), and *Parabacteroides* (Fig 4.12).

For the crude extract fermentation, in comparison with cellulose, significant increases ($P<0.05$) were observed in relative abundance of *Dialister* (Fig. 4.11, note: due to the scale of graph, this data is not clearly visible) and *Parabacteroides* (Fig 4.12), while there was a significant reduction in relative abundance for *Peptostreptococcaceae* (Fig. 4.11), and *Prevotella* (Fig. 4.12). With the depolymerised extract, in comparison with cellulose, significant increases in relative abundance ($P<0.05$) were observed for *Dialister* (Fig. 4.11) for an uncultured genus of *Lachnospiraceae* (Fig. 4.11) and *Parabacteroides* (Fig 4.12), while a significant decrease in the relative abundance of *Bifidobacterium* (Fig. 4.12), was recorded. Between the crude and depolymerisation extracts, significant differences in relative abundance ($P<0.05$) of Gammaproteobacteria B38 (Fig. 4.10), *Alistipes* (Fig. 4.12) and Adlercruetzia (Fig. 4.12) were observed. In comparison with the FOS fermentations, significant increases with the crude extract in relative abundance were observed for *Ruminococcus* (Fig. 4.11), and uncultured Clostridiales bacterium (Fig. 4.11), *Butyricomonas* (Fig 4.12) and *Parabacteroides* (Fig 4.12). A significant decrease ($P>0.05$) in the relative abundance for *Barnsiella* ((Fig. 4.12) was observed.

With the depolymerised extract, in comparison with the FOS fermentations, there were significant increases ($P<0.05$) in the relative abundance of Gammaproteobacteria B38 (Fig. 4.10) *Flavonifractor* (Fig. 4.11), and uncultured *Lachnospiraceae* (Fig. 4.11) with significant decreases in relative abundance of uncultured Clostridiales bacterium (Fig. 4.11) and *Parabacteroides* (Fig 4.12),

4.4.9 Measurement of Alpha (α) and Beta (β) diversity.

Alpha (α)-diversity was measured using Chao1 richness estimation, Shannon's index of diversity, Simpson index of diversity, observed species and phylogenetic diversity metrics (Fig. 4.13). Scatter plot analysis of alpha diversity revealed that supplementation of the fermentation vessels with the extracts had no notable effect on diversity. A slight trend towards reduced diversity was observed overall. Beta (β) diversity was measured using an unweighted Unifrac distance matrix and visualised

in a principle coordinate analysis plot (Fig. 4.14). At 0 h, a trend was observed for the cellulose, FOS, crude extract and depolymerised extract to be clustered close together. At 24 h, clustering was still apparent for the cellulose, crude extract and depolymerised extract, however, for FOS a slight shift occurred away from the cellulose control and both extracts. Visualisations for both extracts remained in close association during all runs.

4.5 Discussion

Seaweeds are widely regarded for their richness of bioactive components but have been overlooked in the main. To date, there have been only a limited number of studies regarding the degradation of seaweeds by the gut microbiota. The objective of this study was firstly to ascertain the prebiotic potential of two polysaccharide extracts prepared from the brown seaweed *L. digitata* using an *ex vivo* fermentation system, and secondly to determine the effect, if any, that depolymerisation had on measurable prebiotic potential.

Certain species of seaweeds are common in the diet of consumers in parts of South East Asia and in Western cultures where it is used in food as emulsifying agent and as thickening agent. Dietary fibre is resistant to digestion and absorption in the small intestine, with partial or total fermentation in the large intestine [30]. Interestingly, most *in vitro* and *in vivo* studies have reported on the low fermentability of seaweed fibre by gut microbes. This is because natural seaweed fibres contain predominantly high molecular weight polymers which then pass through the gut too quickly to be of use as a fermentable substrate by the microbiota [21]. In general, the influence of structure and degree of polymerisation on prebiotic effectiveness of seaweed polysaccharides is not clearly understood. The carbohydrate content of brown seaweeds is high and is estimated to be between 30% and 50%. [31]. Brown seaweeds contain carbohydrates such as laminarin, fucoidan and alginic acid and, as such, a polysaccharide extract prepared from *L. digitata* is likely to be a mix of these polysaccharides.

The extracts used in this study were produced using a dilute hot-acid extraction method, with an additional depolymerisation step included to produce the depolymerised extract. The same raw material was used for both extracts, and during each step of production they were handled and stored in a similar manner. Hydrogen peroxide is both an effective and environmentally friendly oxidant, and has been used to oxidize many polysaccharides such as chitosan, starch, cellulose and dextran. The oxidation method not only depolymerizes the polysaccharide, but also changes the structure of the main chain [32] potentially resulting in an altered fermentation profile. Hydrogen peroxide generates reactive oxygen species, such as HOO^-, HO^-, and O_2^-. These radicals degrade polysaccharides by attacking and breaking glycosidic linkages.

The radical method is mild and the structures of the sugar units are not significantly changed. While this method of depolymerisation is a viable alternative to acid hydrolysis and enzyme treatment for the preparation of low-molecular weight polysaccharides, the issue of radical hydrolysis on the antioxidant activities of sulphated polysaccharides remains [9]. The treatment with hydrogen peroxide results in a depolymerised extract differing from the crude extract in the length of its constituent polysaccharides which were degraded in the depolymerisation process.

Prebiotics selectively stimulate the growth and/or activity of specific bacteria, mainly *Bifidobacterium* and *Lactobacillus*, and thus facilitate a microbiota mediated health effect. The best known prebiotics currently include inulin-type fructans (short-chain FOS (DP 2 - 9) and long-chain inulin (DP 10 - 60); galactooligosaccharides (GOS) and xylooligosaccharides (XOS)), sugar alcohols, resistant starch and complex polysaccharides such as acacia gum [33] Traditionally, the fermentation potential of reputed prebiotics by the microbiota has been evaluated through observing bacterial growth and the production of fermentation end products when pure cultures of intestinal bacteria (in particular bifidobacteria and lactobacilli) are grown on selective media in their presence. Most of these prebiotics have resulted in increased numbers of bifidobacteria recovered in faeces [34]. The chemical structure of laminarin consists mainly of a linear β-(1→3)-linked glucan with some random β-(1→6)-linked side chains, depending on the type of seaweed. β-Glucans have been shown to exhibit prebiotic properties by increasing the number of *Bifidobacterium* and *Lactobacillus* in the pig colon [35]

The major microbial fermentation products stemming from prebiotic metabolism in the large colon are SCFAs [36]. Laminarin and fucoidan, as well as other polysaccharides, undergo microbial fermentation in the cecum resulting in the production of multiple groups of metabolites, with SCFA being the foremost group [37]. Changes in SCFA production patterns are a strong indication of stimulation of growth and/or activity of the microbiota. This is one of the central tenents of the prebiotic concept. The known prebiotic FOS is resistant to degradation by human enzymes in the small intestine, but is extensively fermented in the large bowel to SCFAs, which are absorbed and further metabolised by the host [36] and members of the microbiota. A study carried out using FOS and inulin of differing degrees of

polymerisation demonstrated that the shorter-chain FOS were more rapidly fermented than long-chain inulin which were more steadily fermented [38]. The dietary supplementation of porcine diet with laminarin/fucoidan has been reported to induce increased SCFA production[37]. As expected, the addition of FOS, the crude extract and the depolymerised extract to their respective fermentation vessels in the current study resulted in rapid and significant increases in both the total concentration of SCFAs and the rate of their production in comparison with the negative control. The FOS achieved the highest absolute concentration of SCFAs. The final absolute concentration of SCFA produced by both *L. digitata* extracts was comparable to that of FOS, however SCFA concentration with the crude extract was significantly ($P<0.05$) lower than the FOS at every previous timepoint studied. There was no significant difference observed in SCFA concentrations between the depolymerised extract and the FOS control except at 5 h. Both *L. digitata* extracts underwent a lag phase in SCFA production at the beginning of the fermentation. It has been reported that such a delay is required by the microbiota to synthesise the enzymes necessary to hydrolyse and metabolise the polysaccharides present in the extracts [11]. FOS consists of mixtures of fructose moieties linked by β-(2→1)-glycosidic bonds to a terminal glucose unit [39]. Bonds of this configuration are easily cleaved by enzymes produced by the microbiota. The FOS positive control used in this body of work has an average dp of less than 10

All fermentations produced significantly more SCFA than the poorly fermentable cellulose control. The timeline of SCFA production indicates that both *L. digitata* extracts and FOS were readily fermented by the microbiota with most SCFA production taking place during the first 10 hours. For the FOS fermentation, SCFA production was observed to peak during the initial five hours of the fermentation while SCFA production for both extracts reached its zenith between 5 h and 10 h. The production profile for the FOS agreed with previous data, which had shown significant increases in production of the three biologically significant SCFAs, acetate, butyrate and propionate. Importantly, both the crude *L. digitata* and the depolymerised extract also gave rise to significant increases in production of these SCFAs. The production of acetate, propionate and butyrate in the human colon is highly dynamic. For example, both butyrate and propionate can be degraded to acetate (in the absence of nitrate or sulphate) or be completely oxidized to molecular oxygen and dioxide by

nitrate-reducing or sulphate reducing bacteria [40]. Acetate was the major SCFA produced in each of the fermentation runs, accounting for approx. 50% of all SCFAs produced. A significant increase in acetate concentration was observed for all fermentation conditions in comparison with the cellulose control. Acetate production with the FOS fermentation occurred quickly following inoculation of the vessels with more than 70% being produced in the first 5 hours. Acetate production for both *L. digital* extracts was slower and showed rather a steady level of production during the first 24 h of the fermentation, with approx. 75% of acetate in the crude extract fermentation and 85% of the acetate by the depolymerised extract being produced by 24 h. The depolymerisation of *L. digitata* had no significant effect on the production of acetate. Both *L. digitata* extracts exhibited a similar acetate profile and there were no significant differences in either rate of production or in final concentration. Acetate serves as an energy source for the liver and peripheral tissues and also acts as a signaling molecule in metabolic pathways of gluconeogenesis and lipogenesis [19]. The second major SCFA of biological important produced during the fermentations was propionate. As with acetate, anaerobic microbial fermentation of the FOS control and both seaweed extracts generated significant increases in propionate concentration and production. Propionate production in the presence of the depolymerised extract, during the initial hours of the fermentation, was significantly ($P<0.05$) increased over that observed for the crude extract. Polysaccharide chain length is an important factor in microbial fermentation patterns and in this study the depolymerisation of *L. digitata* had a significant positive impact on the production of propionate. The shorter chain polysaccharides present in the depolymerised extract favoured the production of propionate by the representative microbial community. Butyrate was the third most abundant SCFA produced during the fermentations. For all experimental conditions, butyrate concentration and rate of production was significantly increased over that of the cellulose control. An increased level of butyrate production was observed during the fermentation of both *L. digitata* extracts and would suggest that both extracts were successful in stimulating butyrate producers in the representative microbiota. The total production of butyrate was significantly ($P<0.05$) greater for the crude extract than the depolymerised extract. The native polysaccharides of the crude extract favoured the production of butyrate over the shorter-chained polysaccharides found of the depolymerised extract. This observation agrees with studies that have shown that long-

chain inulin enhances the production of butyrate by the microbiota. Bifidobacteria, however, do not produce butyrate leaving the question of how this comes about somewhat unclear. It is likely that bacterial cross feeding plays a part of this enhancement of butyrate production [34]. The production of butyrate by mixed human faecal microbiota *in vitro* is strongly influenced by the growth substance. Starch is an example of a strongly butyrogenic molecule whereas pectin is relatively less so and leads more to acetate and propionate production [41]

The prebiotic definition states that the stimulation of the gut microbiota is a key aspect of any putative prebiotic. As SCFAs are the main fermentation products of the microbiota, an increase in their production would be an indicative sign that the fermentation substrate has prebiotic potential. Of the three biologically significant SCFAs (acetate, propionate and butyrate) butyrate and propionate are of most interest, however butyrate production attracts the most attention due to its antineoplastic properties and other beneficial biological functions in the colon [21, 36]. Butyrate has been shown to downregulate innate responses in various biological systems [42]; Butyrate is proposed to play a key role in maintaining gut homeostasis and epithelial integrity as it directly influences host genome expression by inhibiting histone deacetylases, and interferes with proinflammatory signals such as NF-κβ. Butyrate is synthesised via pyruvate and acetyl-coenzyme A (CoA), mostly by the breakdown of complex polysaccharides that reach the colon intact after passing through the upper GIT. Alternative substrates can be derived from cross-feeding with other primary degraders and lactate-synthesizing bacteria. Acetyl-CoA is then converted to the intermediary butyryl CoA in a manner closely related to β-oxidation of fatty acids [43]. Butyrate is a major energy source for colonocytes and is taken up either by passive diffusion or via apical uptake transporters such as monocarboxylate transporter 1 (MCT1) and sodium coupled monocarboxylate transporter 1 (SMCT1). Butyric acid is reported to reduce the risk of colon cancer, protect the mucosa through stimulation of mucus secretion and tight junction integrity, and acts as an anti-inflammatory agent. It also increases the expression of MCT1 and its chaperone CD147 [44]. The ability to produce butyrate is widely distributed among Gram-positive anaerobic bacteria that colonize the human gut [45]. Several species within families belonging to the phylum *Firmicutes* have been identified as butyrate producers. The most abundant groups found in healthy adult faecal matter are bacteria

related to *Roseburia* spp. (family *Lachnospiraceae*), *Eubacterium rectale* (family *Lachnospiraceae*) and *Faecalibacterium prausnitzii*-related bacteria (family *Clostridaceae*) [40].

SCFA concentration at each timepoint during the depolymerised extract fermentation was on average higher than that of the crude extract at all timepoint except at 48 h; however, there was no significant ($P<0.05$) difference in their respective concentrations at any timepoint. One key influence that fermentable substrates can have on SCFA production is on the relative ratio of individual fatty acids produced. Substrates that shift the balance to beneficial SCFA are favoured over those that lead to the increased fermentation of proteins and which produce toxic substances. The ratio and extent of SCFA production is a complex interplay between substrate type, microbiome diversity and activity. Acetate, propionate and butyrate, taking evidence from available human studies, are present in the approximate molar ratio of 60:20:18 [46]. The other principal end products of colonic fermentation are the gases hydrogen, carbon dioxide and methane with small quantities of branched chain fatty acid (iso-butyrate, valerate, iso-valerate and hexanoate), which are formed from protein and amino degradation [47]. Molar ratios of the primary SCFA in this study further indicate that fermentation of the crude extract resulted in a shift towards the production of butyrate at the expense of acetate and propionate.

DNA sequencing making use of 16s rRNA gene is the gold standard for microbial identification. Of the 52 currently recognised bacterial phyla, approximately five to seven phyla are known to be resident in the mammalian GIT. Generally, the phyla Firmicutes and Bacteroidetes dominate the gut microbial community while members of the Proteobacteria, Actinobacteria, Verrucomicrobia and the new candidate phylum *TM7* are less abundant. Despite this relatively small representation of dominant phyla, the number of bacterial species is estimated to be over a thousand, and the number of the genes is more than 150-fold greater than the number of genes in their hosts [48]. Clearly, the modulation of the gut microbiota towards a desirable composition is of great importance in terms of prebiotic studies. Phylogenetic data obtained during this study broadly agreed with data reported in other studies in terms of the composition of the dominant phyla, families and genera of the representative microbiota. The dominant phyla were the Firmicutes and Bacteroidetes, with large

numbers of Actinobacteria and Proteobacteria also present. The effect of FOS on the microbial community was a non-significant increase in the relative abundance of Actinobacteria and Proteobacteria and a decrease in the relative abundance of Bacteroidetes and Firmicutes. The relative increase in Proteobacteria was small and significantly lower than that of both the cellulose control and the crude extract, which both recorded relatively large increases in Proteobacteria abundance. The Proteobacteria comprise a large grouping of Gram-negative bacteria including many pathogens, whose growth would ideally be inhibited to promote a good GIT environment. Based on results obtained for bacteria at the phylum level of organisation, depolymerisation had no influence on the relative abundance of any bacterial phylum.

Neither of the *L. digitata* extracts had any significant effect on the relative abundance of *Bifidobacterium* (phylum Firmicutes, family Bifidobacteriaceae) or *Lactobacillus* (phylum Firmicutes, family Lactobacillaceae). Both bifidobacteria and lactobacilli are known to be beneficial bacteria of the human microbiota [11] and positive changes in their relative abundance would have been expected for a putative prebiotic. Previous studies have reported increased numbers of bifidobacteria and lactobacilli in the presence of inulin and other non-digestible oligosaccharides [21]. Bifidobacterial enumeration on selective media also indicated that there was no stimulation of growth by either extract. This would indicate that the *L. digitata* extracts are not bifidogenic in their current form. There could be two reasons for this. Firstly, bifidobacteria and lactobacilli are unable to metabolise polysaccharides from *L. digitata* through carbohydrate utilisation pathways such as the fructose-6-phosphate phosphoketolase (F6PPK) pathway and, secondly, the extracts contain some biologically active component that inhibits the growth of bifidobacteria and lactobacilli. Further, the depolymerisation of *L. digitata* had no significant bearing on the relative abundance of bifidobacteria and lactobacilli or on the recovery of culturable *Bifidobacterium* or *Lactobacillus*. This agrees with the observations of a recent animal trial where the supplementary addition of laminarin to porcine diet also had no effect on the recovered faecal *Bifidobacterium* populations [49]. Another study reported that laminarin exhibits antimicrobial properties and has been reported to reduce populations of *Enterobacterium*, *Bifidobacterium* and *Lactobacillus* in the caecum and colon of pigs that were fed a *Laminaria*-derived seaweed extract [37].

Diversity within the adult faecal microbiota was visualised using scatter plot analysis of α-diversity and unweighted PCoA (Principle co-ordinate Analysis) plots of β-diversity. Fermentation of both *L. digitata* extract had limited impact on the diversity within the microbial community. The PCoA of β-diversity indicated a clear separation between microbial communities based on fermentation run rather than treatment condition. A similar effect was also previously seen (Chapter 3) involving the fermentation of a *Fucus serratus* polysaccharide extract. The FOS fermentation had a notable effect on diversity compared with the cellulose control and both *L. digitata* extracts.

Sequencing data for the FOS control also failed to detect any bifidogenic activity at 24 h; however, plate counts on selective agar indicated that FOS supplementation significantly ($P<0.005$) increased culturable *Bifidobacterium* numbers by more than 0.5 log in comparison with the cellulose control, and both *L. digitata* extracts at 10 h but not at 5 h or 24 h. Each fermentation system is a closed system, that is, there was no addition of extra fermentable material over the course of the fermentation run nor was there a removal of bacterial by-products. Plate count data suggest that after 10 h, *Bifidobacterium* become starved of nutrients and/or were outcompeted by other surviving bacteria resulting in a reduction in their number by 24 h. The crude extract and the depolymerised extract exhibited similar effects during their respective fermentations. At the family level, both extracts promoted the growth of *Lachnospiraceae* (phylum Firmicutes, class Clostridia) and *Erysipelotrichaceae* (phylum Firmicutes) in comparison with the cellulose control. Lachnospiraceae is an abundant family of anaerobic bacteria found in the mammalian digestive tract and relatively rare elsewhere. Members of this family have been linked to obesity and the protection of humans from colon cancer mainly owing to an association of several members of the family with the production of butyrate [50]. The family *Erysipelotrichaceae* is reported to have a potential role in host physiology and/or disease. These organisms appear to be highly immunogenic and can potentially flourish following treatment with broad spectrum antibiotics; however, studies examining the direct impact of changes in their abundance on host health are required [51]. At the genus level, both extracts promoted the growth of *Parabacteroides* (phylum Bacteroidetes, family *Porphyromonadaceae*), Gram-negative, obligately anaerobic non-motile rods whose major fermentation end products are acetate and

succinate [52], and *Dialister* (phylum Firmicutes, family *Veillonellaceae*) which are anaerobic or microaerophilic Gram-negative cocci [53]. One difference of note between the two extracts was that the crude extract did significantly impact upon the abundance of the genus *Alistipes* (phylum Bacteroidetes, family *Rikenellaceae*) in comparison with the depolymerised extract. This genus of bacteria is Gram-negative, strictly anaerobic, rod shaped bacteria that produce succinate as their principle metabolic end-product of glucose fermentation [54]. Based on the results obtained from direct enumeration of culturable bacteria and 16s rDNA sequencing, the depolymerisation of *L. digitata* by hydrogen peroxide was seen to have a negligible overall impact on faecal microbiota composition underlying their common origin.

This study has shown that polysaccharides from the brown seaweed *L. digitata* can survive a simulated gastric challenge and are readily fermentable by intestinal bacteria. The fermentation of both *L. digitata* extracts resulted in significant increases in production of the biologically significant SCFAs butyrate, propionate and acetate. However, plate count data and DNA sequencing results yielded no evidence to suggest that *L. digitata* polysaccharides have any stimulatory effect on *Bifidobacterium* or *Lactobacillus.* These genera represent the main targets of prebiotic and probiotic studies and because of this we cannot confirm prebiotic activity of either extract in their current form or under the current definition of a prebiotic. Here, we have demonstrated *L. digitata* to be an excellent source of dietary fibre that can modulate the activity of human gut bacteria. The crude *L. digitata* extract, possessing the parent polysaccharides, generated a significantly increased amount of butyrate, compared to the depolymerised extract. Butyrate is a preferred energy source for colonic epithelial cells and is thought to play an important role in maintaining colonic health in humans while also exhibiting anti-tumour activity. Increased production of butyrate by the colonic microbiota is a desirable result of anaerobic fermentation. Importantly, it was found that depolymerisation of *L. digitata* polysaccharides had a significant impact on fermentation outcomes. Fermentation of the shorter chain polysaccharide of the depolymerised extract resulted in a shift in SCFA production to favour propionate over butyrate. Propionate has been heavily linked with satiety and an increase in its production could lead to positive result outcomes in terms of obesity.

4.6 Conclusion.

In conclusion, through modulation of polysaccharide chain length by depolymerisation processes, different fermentation profiles can be obtained from the same seaweed raw material and starting faecal microbiota. This could potentially allow ‘designer’ extracts to be produced, whose impact on the gut microbiota can be predicted beforehand. The definitive test of the prebiotic potential of a substrate is to transition from *ex vivo* to *in vivo* animal trials in small animals and subsequently in large animals/humans. However, several challenges exist in the upscaling of prebiotic studies. As the yield of polysaccharides can be very low following acid hydrolysis, large quantities of seaweed would need to be collected, stored and processed to produce sufficient extracts for further investigation. Additionally, the concentration and type of seaweed polysaccharide can vary greatly depending on the season of collection and the species of seaweed and must be taken into consideration in the production of the product. Fermentation of *L. digitata* polysaccharides did not result in confirmation of prebiotic activity, however, positive changes in SCFA production indicate that this seaweed would be an ideal candidate for *in vivo* prebiotic trials.

4.7 References.

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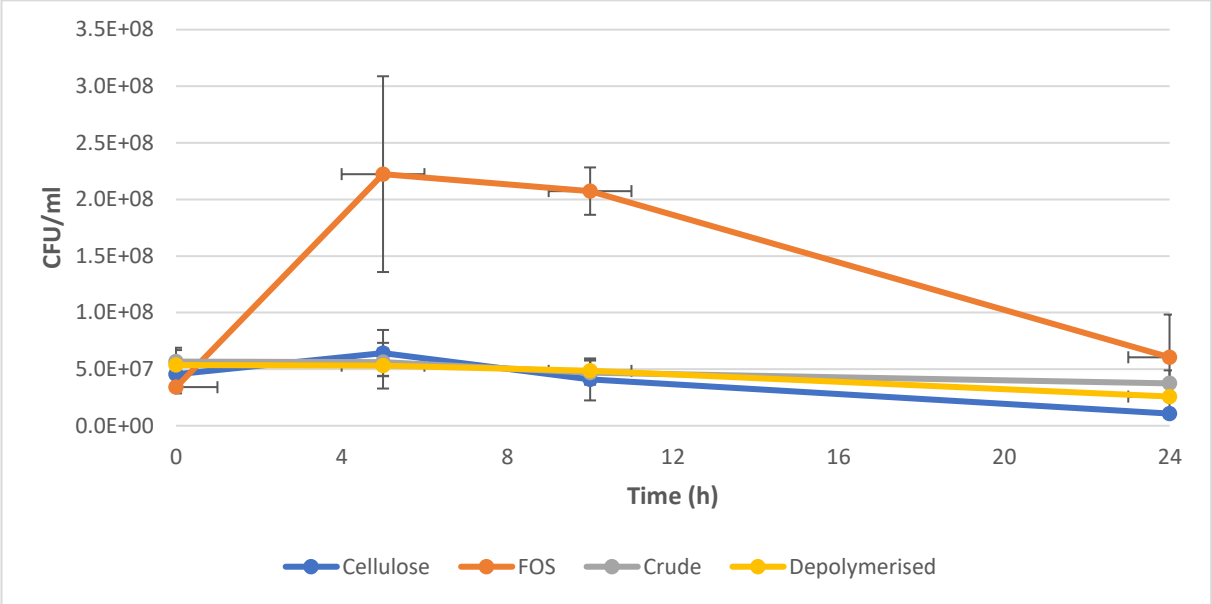
Figure 4.1. Enumeration of a) *Bifidobacterium* and (b) *Lactobacillus*. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose)

(a)



(b)

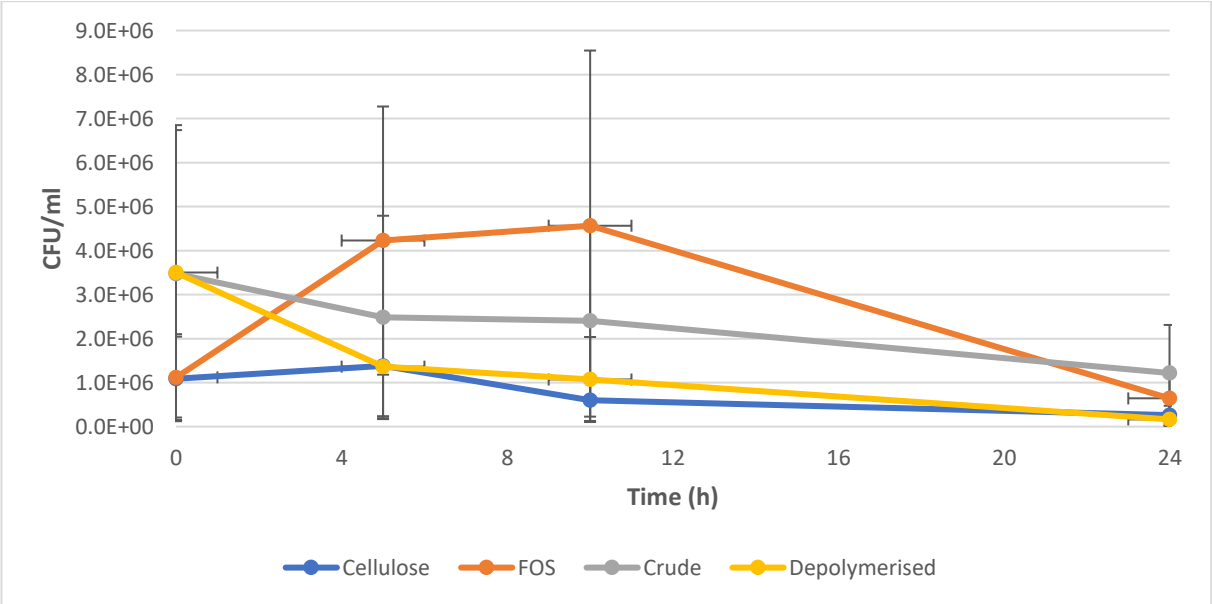
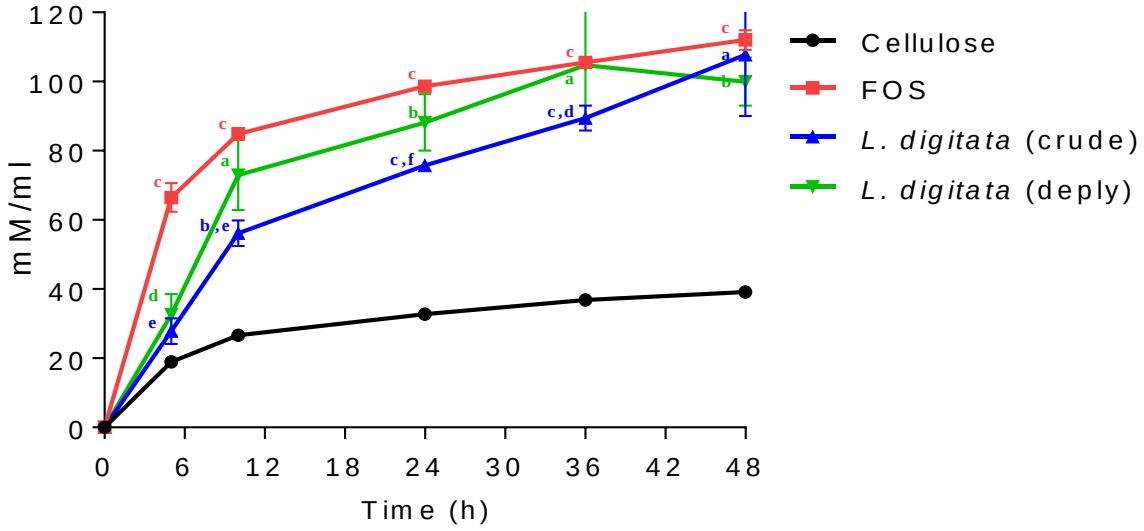


Figure 4.2. The effect of fermentation on (a) Total SCFA concentration and (b) SCFA production per timepoint. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test). Red denotes significance for FOS, Blue denotes significance for *L. digitata* crude extract, and Green significance for denotes *L. digitata* depolymerised extract.

(a)



(b)

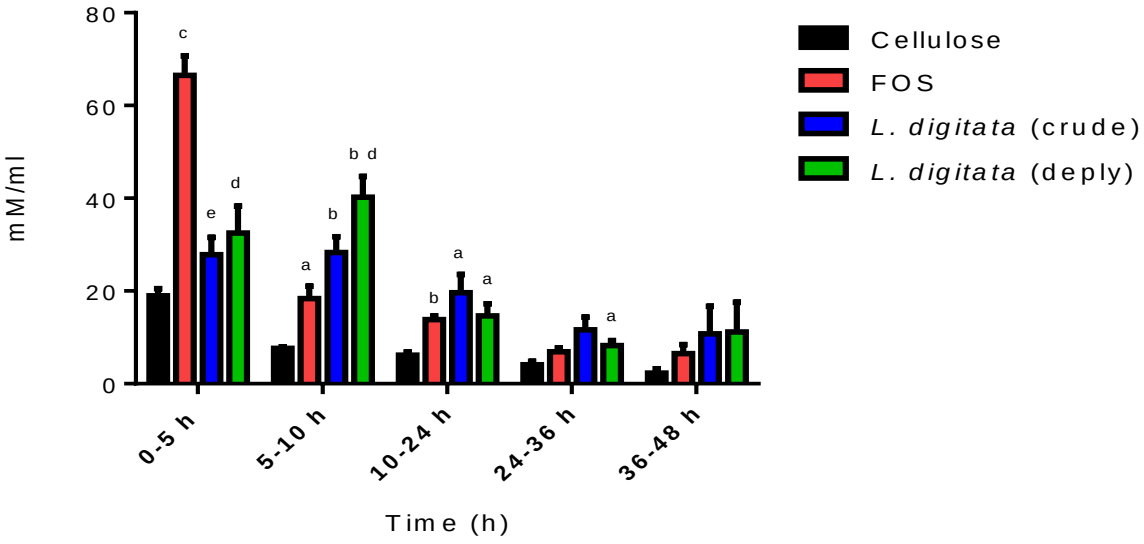
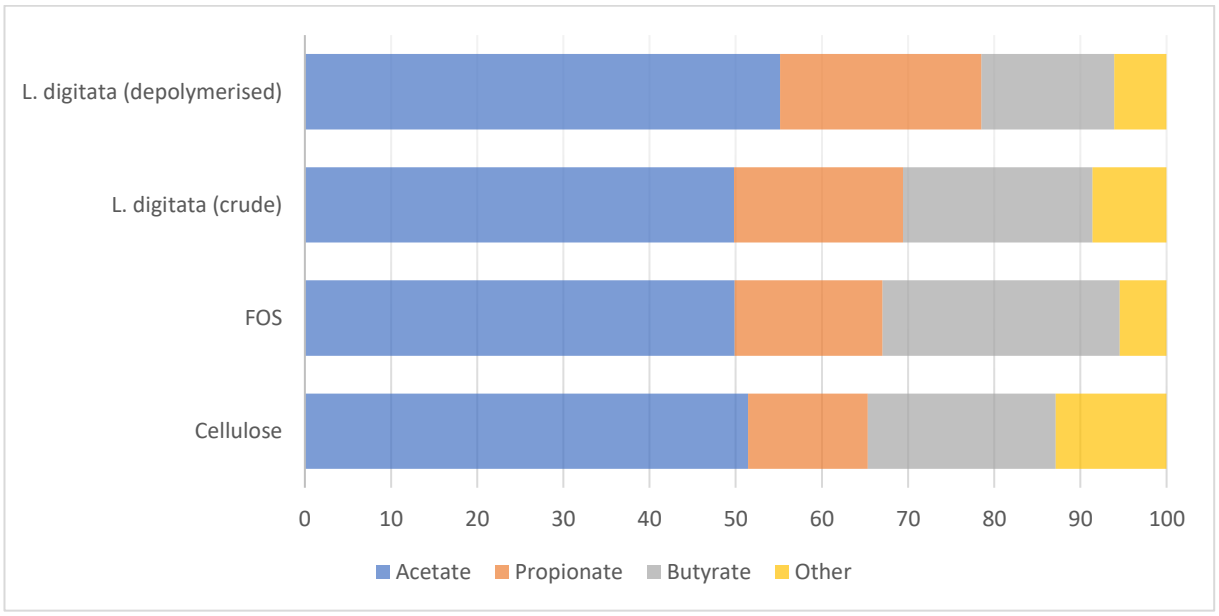


Figure 4.3. The percentage breakdown of total SCFA production. Breakdown of SCFA production (a) Total and (b) between 0 – 24 h.

(a) Total production



(b) Production between 0 – 24 h

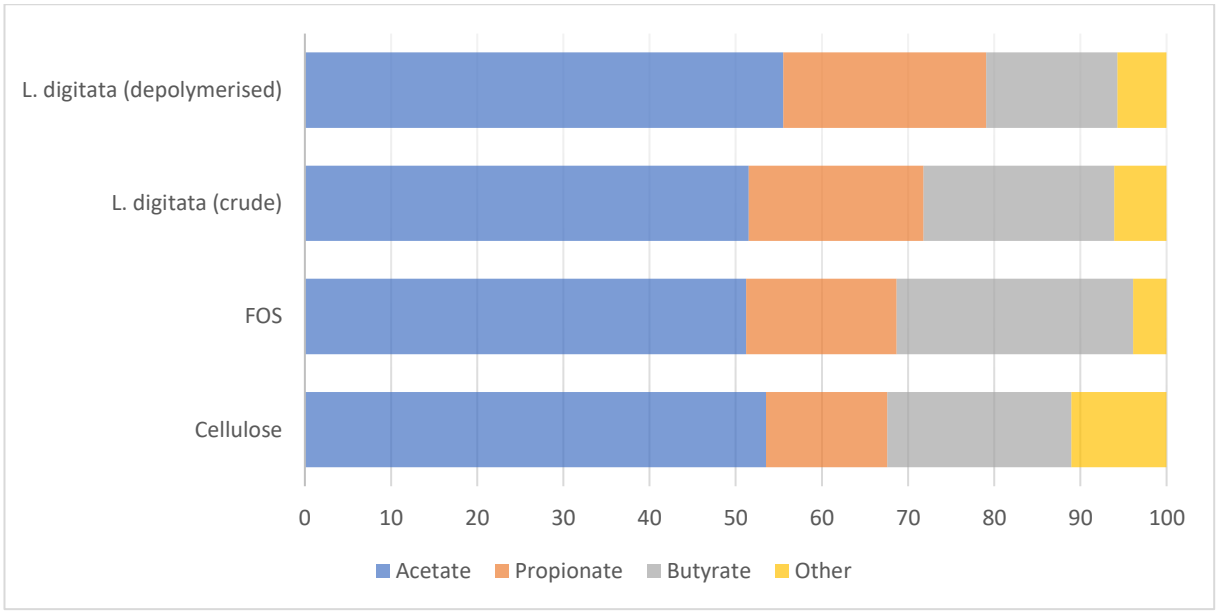
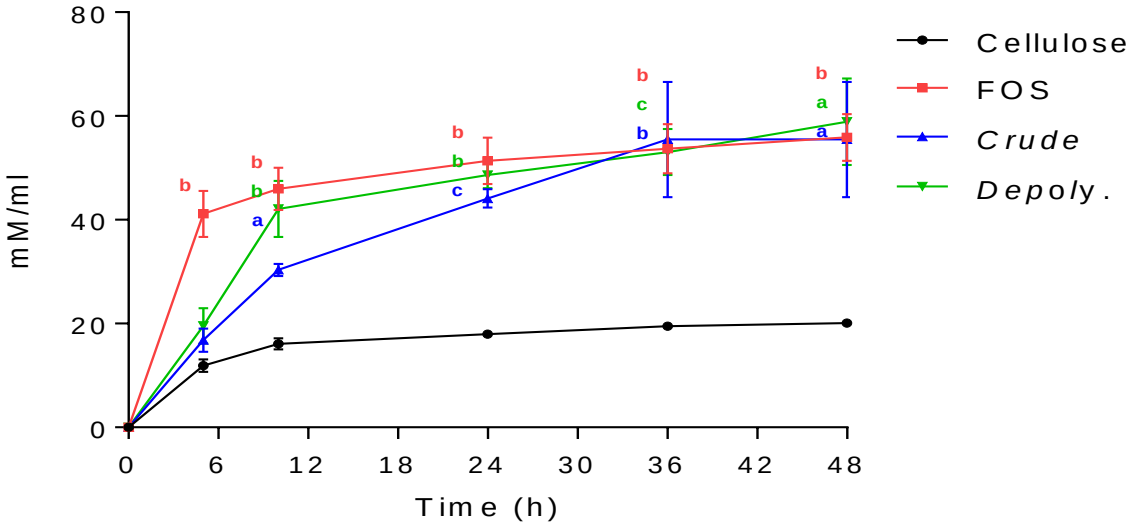


Figure 4.4. The effect of fermentation on (a) Acetate concentration and (b) Acetate production per timepoint. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test). Red denotes significance for FOS, Blue denotes significance for *L. digitata* crude extract, and Green significance for denotes *L. digitata* depolymerised extract.

(a)



(b)

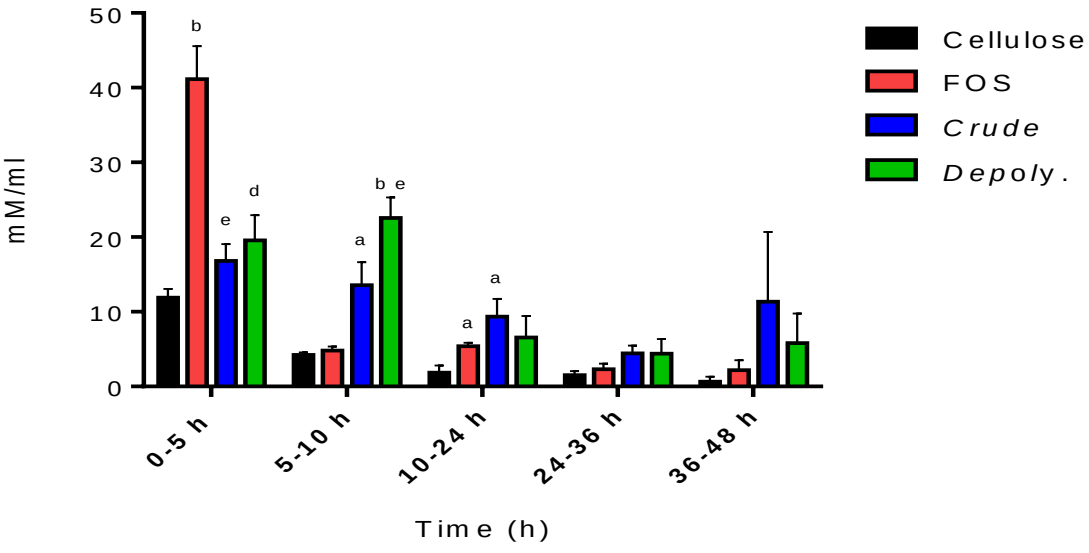
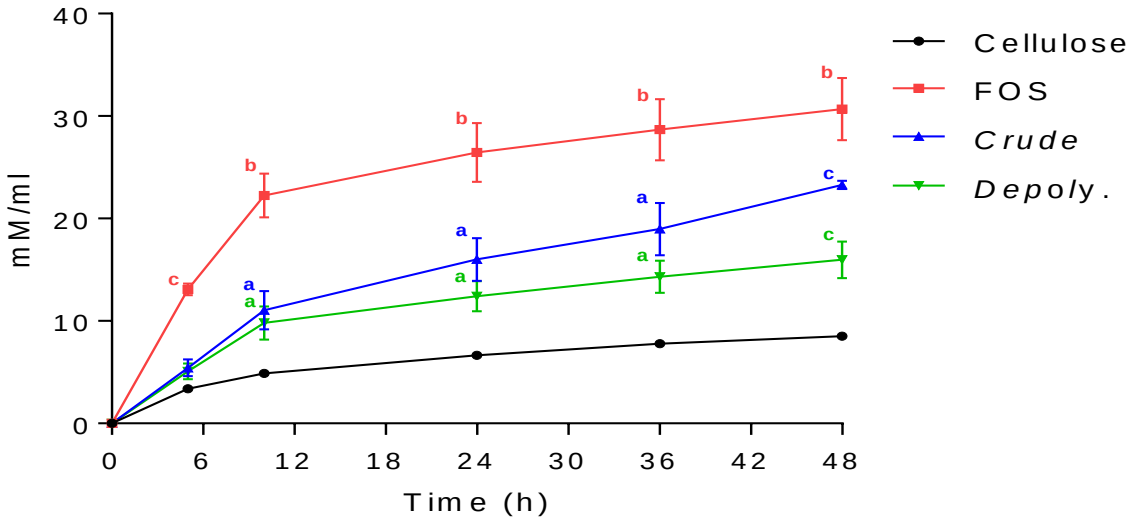


Figure 4.5. The effect of fermentation on (a) Butyrate concentration and (b) Butyrate production per timepoint. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test). Red denotes significance for FOS, Blue denotes significance for *L. digitata* crude extract, and Green significance for denotes *L. digitata* depolymerised extract.

(a)



(b)

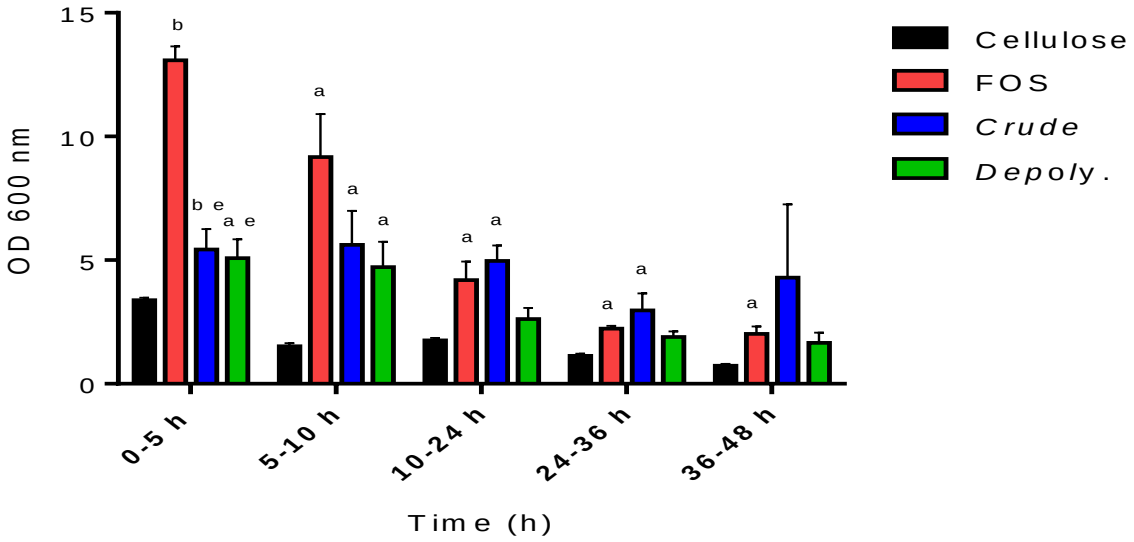
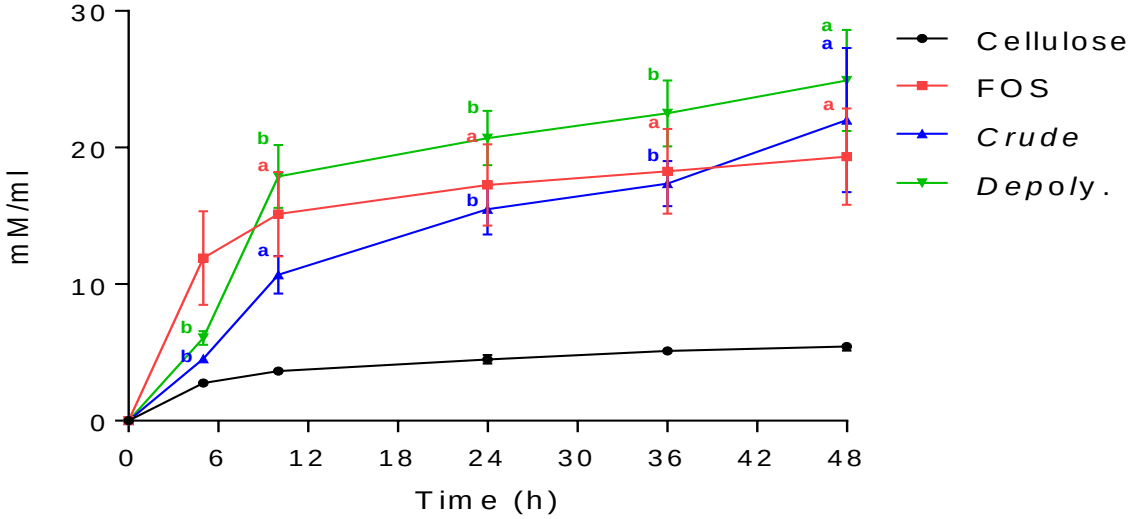


Figure 4.6. The effect of fermentation on (a) Propionate concentration and (b) Propionate production per timepoint. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test). Red denotes significance for FOS, Blue denotes significance for *L. digitata* crude extract, and Green significance for denotes *L. digitata* depolymerised extract.

(a)



(b)

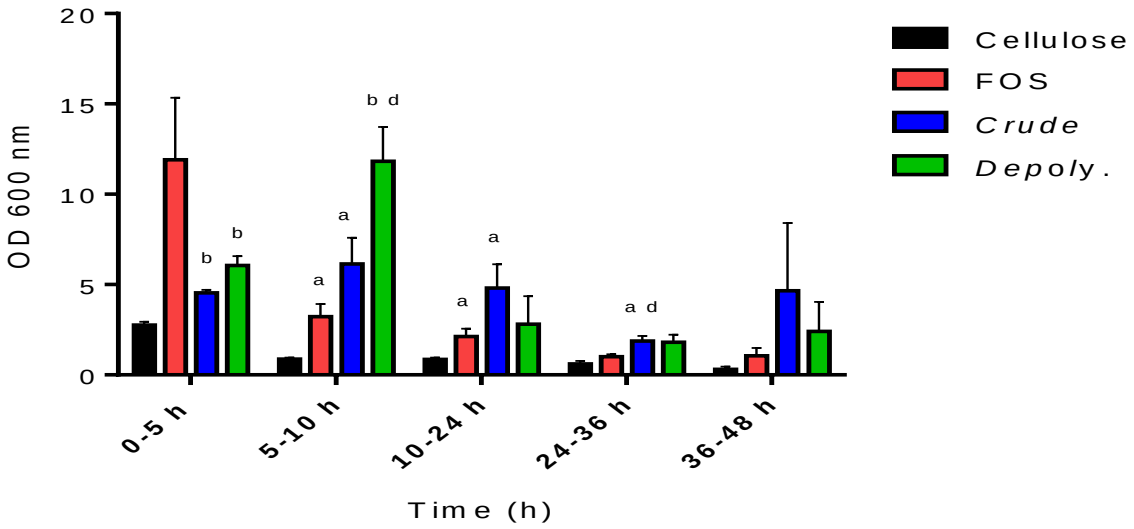
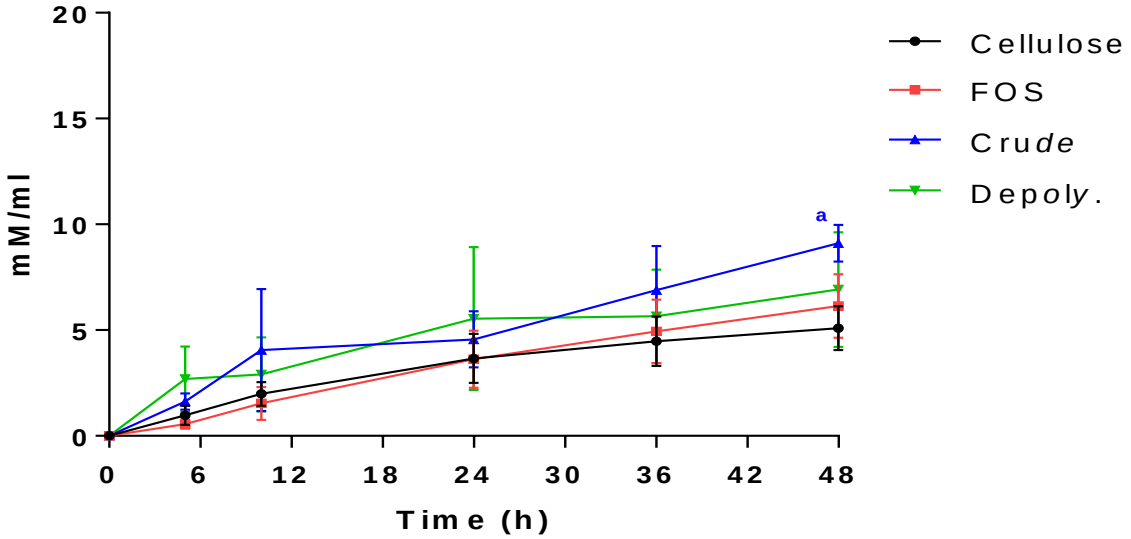


Figure 4.7. The effect of fermentation on (a) BCFA concentration and (b) BCFA production per timepoint. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test). Red denotes significance for FOS, Blue denotes significance for *L. digitata* crude extract, and Green significance for denotes *L. digitata* depolymerised extract.

(a)



(b)

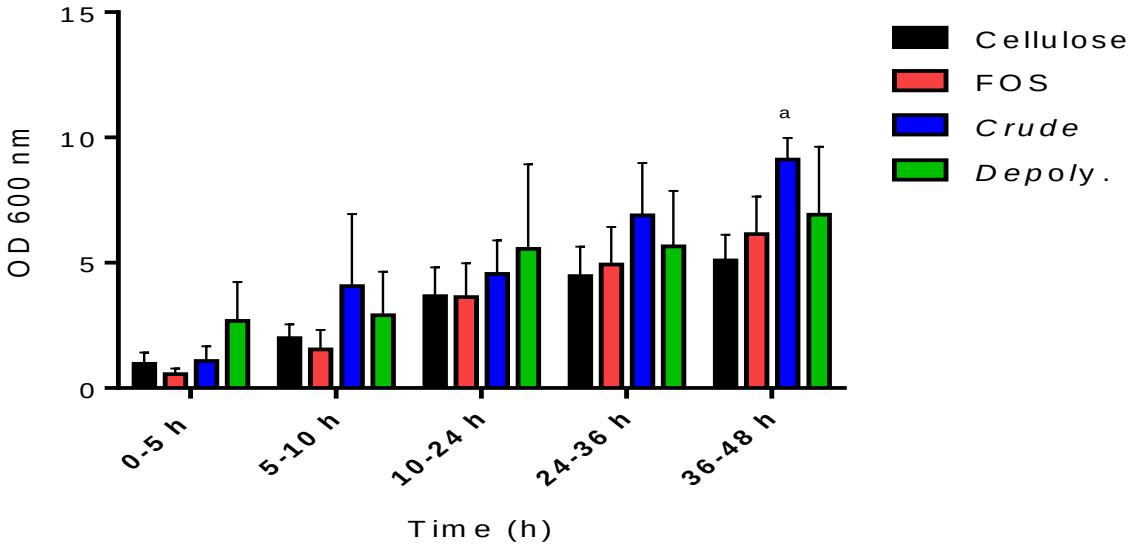
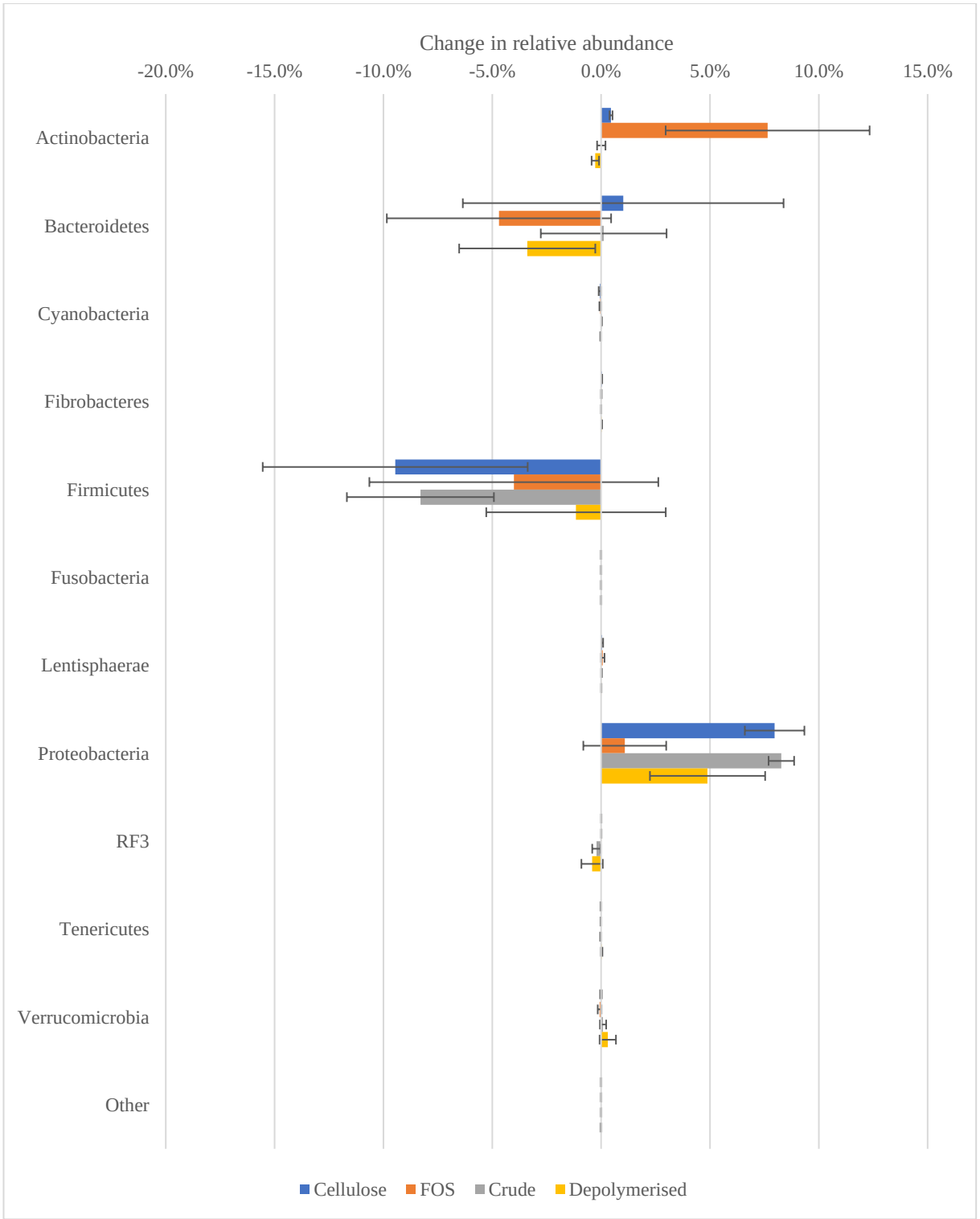


Figure 4.8 (a) Increase/decrease in relative abundance at the phylum level. (b) Percentage change in relative abundance at the phylum level Data represent the mean (±SE).

(a)



(b)

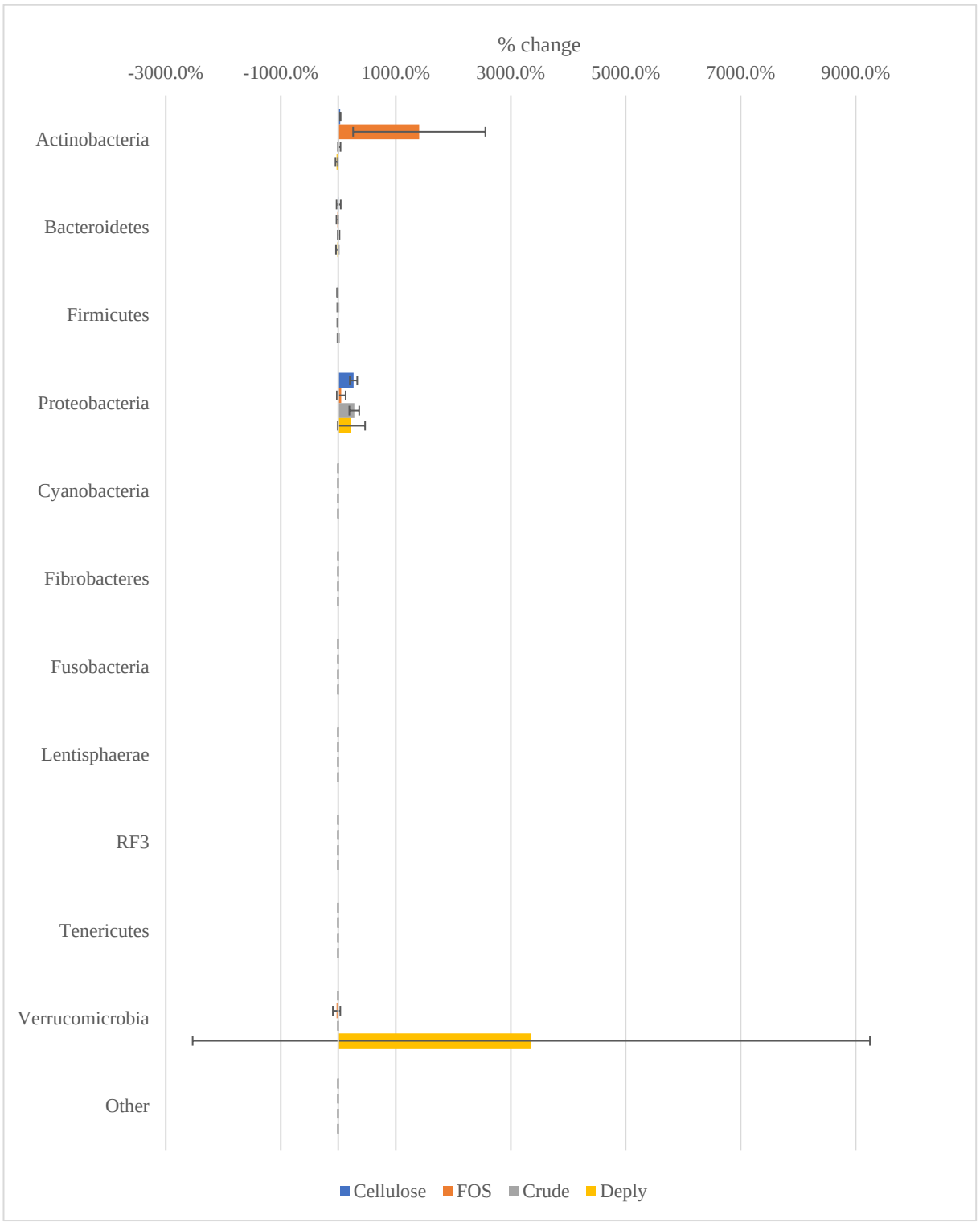
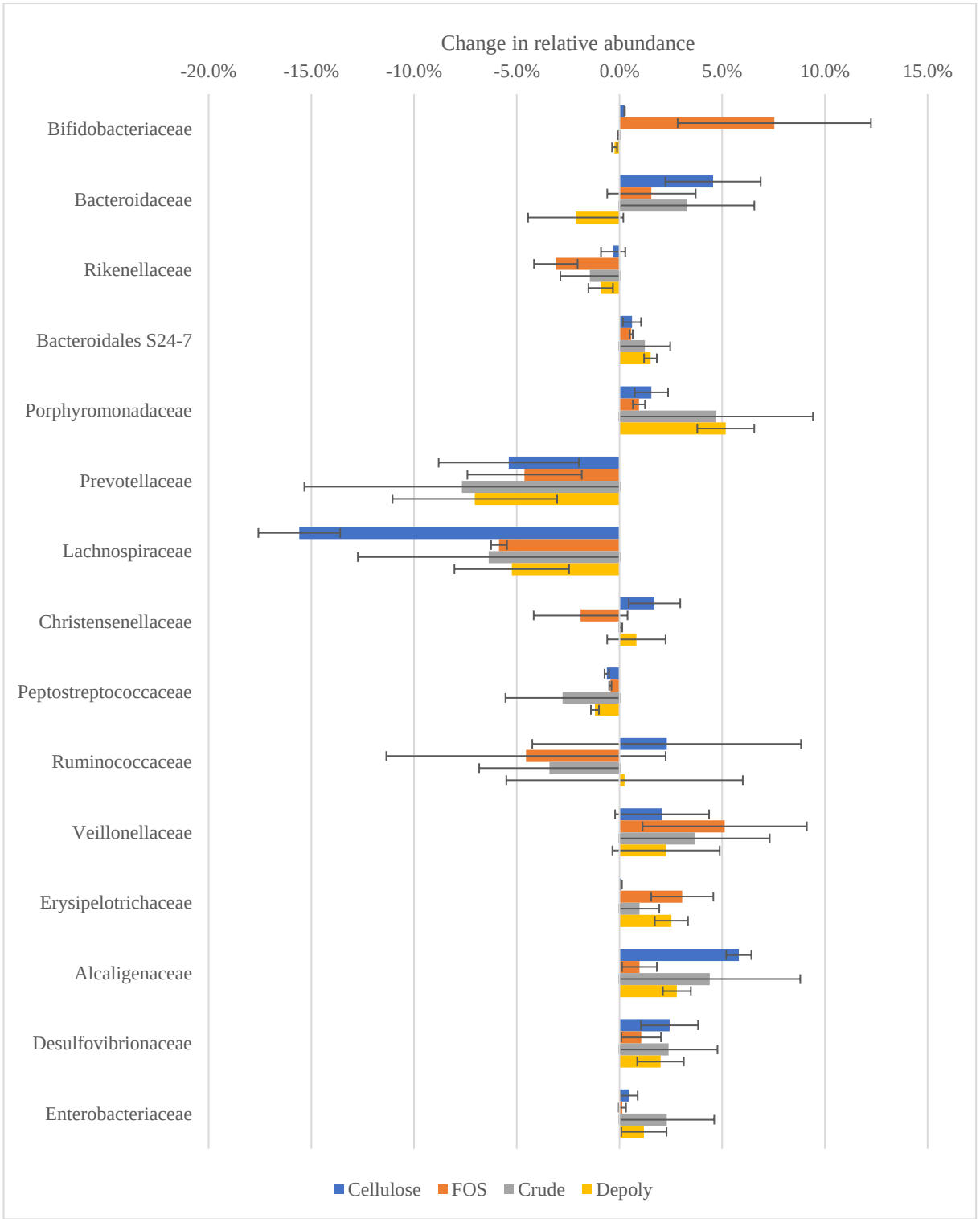
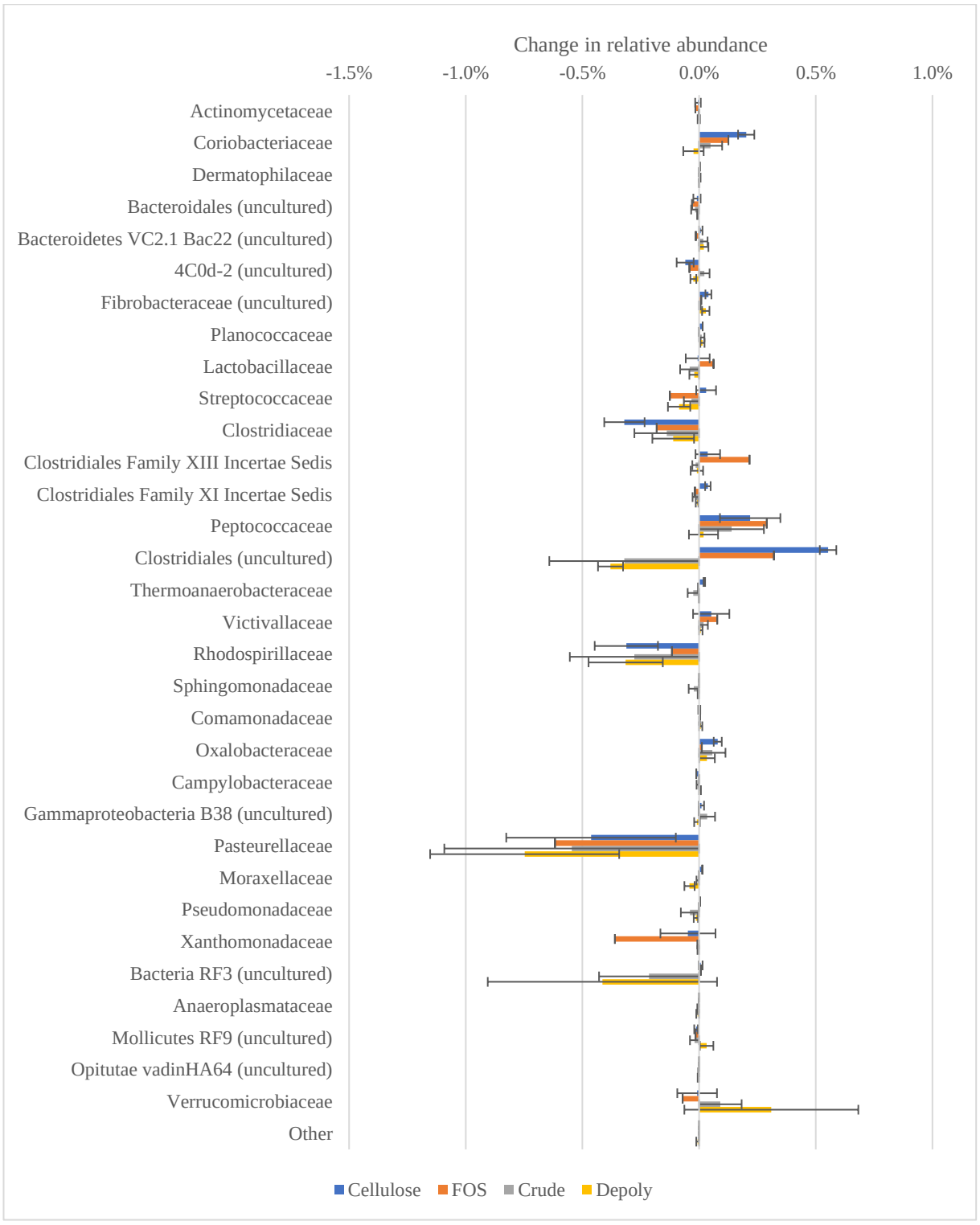

% change
-3000.0%
-1000.0%
1000.0%
3000.0%
5000.0%
7000.0%
9000.0%
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Cyanobacteria
Fibrobacteres
Fusobacteria
Lentisphaerae
RF3
Tenericutes
Verrucomicrobia
Other
Cellulose
FOS
Crude
Deply

Figure 4.9 (a) Increase/decrease in relative abundance at the family level. (b) Percentage change in the relative abundance at the family level. Values represent the mean (±SE).

(a)




Change in relative abundance
-1.5%
-1.0%
-0.5%
0.0%
0.5%
1.0%
Actinomycetaceae
Coriobacteriaceae
Dermatophilaceae
Bacteroidales (uncultured)
Bacteroidetes VC2.1 Bac22 (uncultured)
4C0d-2 (uncultured)
Fibrobacteraceae (uncultured)
Planococcaceae
Lactobacillaceae
Streptococcaceae
Clostridiaceae
Clostridiales Family XIII Incertae Sedis
Clostridiales Family XI Incertae Sedis
Peptococcaceae
Clostridiales (uncultured)
Thermoanaerobacteraceae
Victivallaceae
Rhodospirillaceae
Sphingomonadaceae
Comamonadaceae
Oxalobacteraceae
Campylobacteraceae
Gammaproteobacteria B38 (uncultured)
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Xanthomonadaceae
Bacteria RF3 (uncultured)
Anaeroplasmataceae
Mollicutes RF9 (uncultured)
Opitutae vadinHA64 (uncultured)
Verrucomicrobiaceae
Other
Cellulose
FOS
Crude
Depoly

(b)

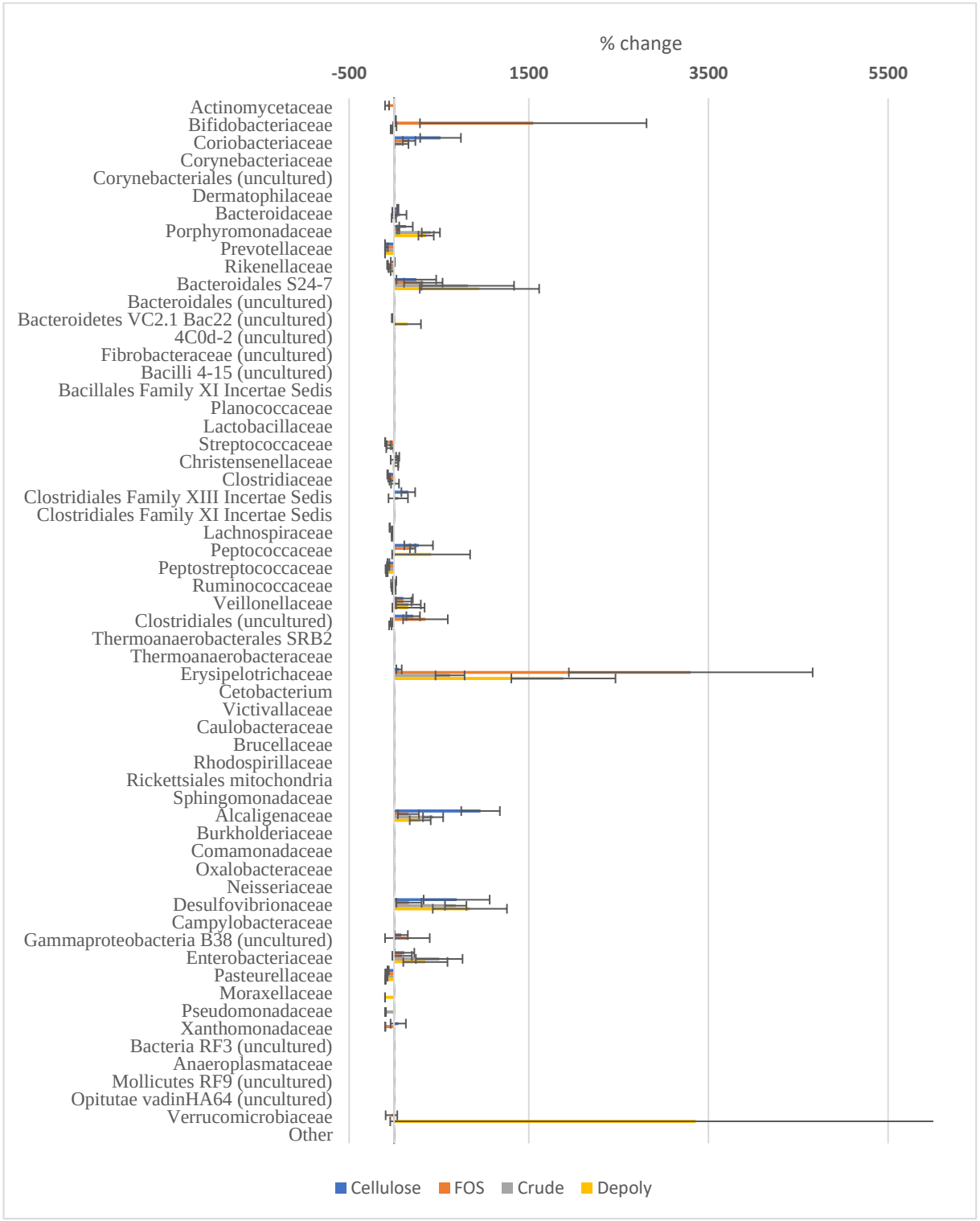
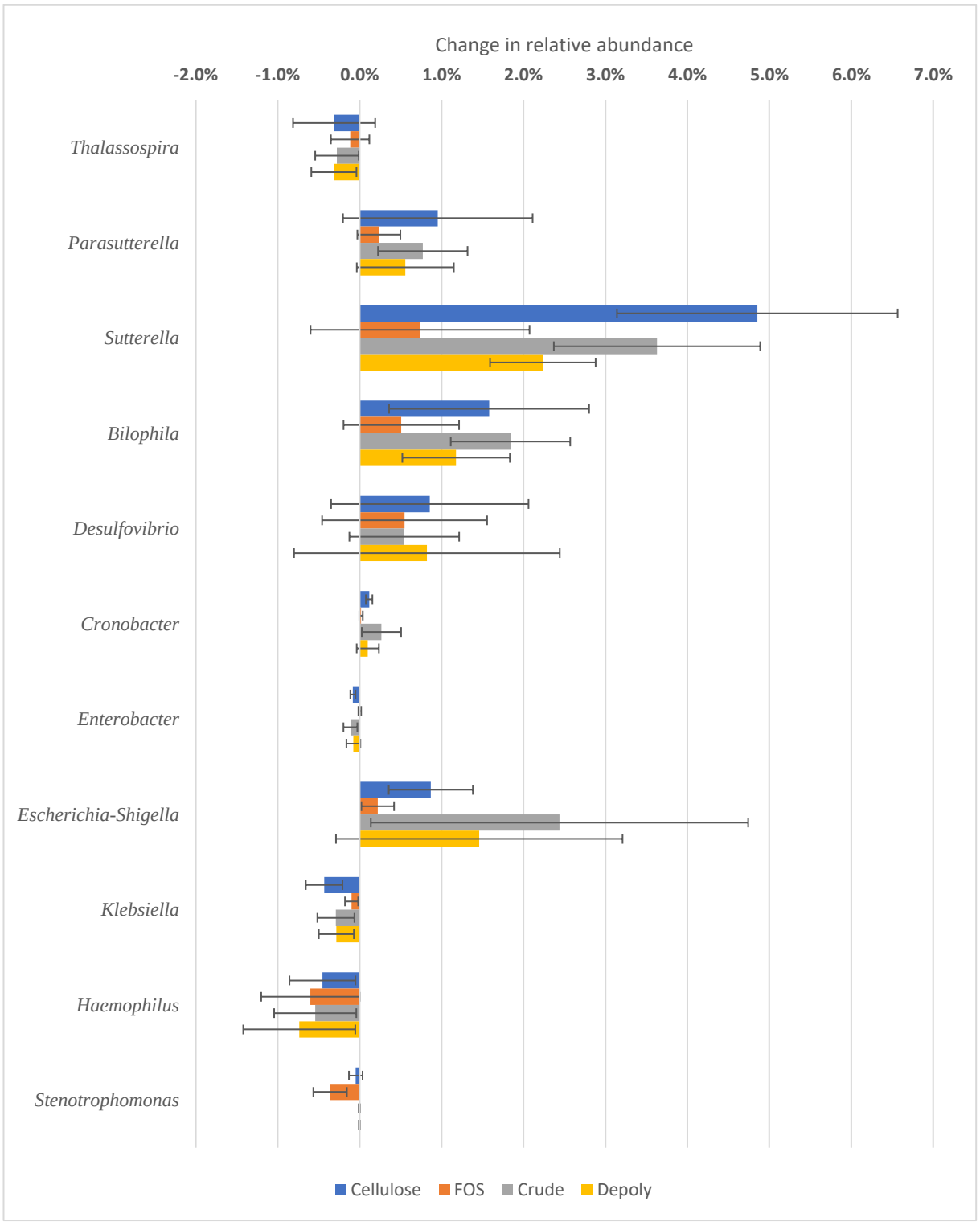
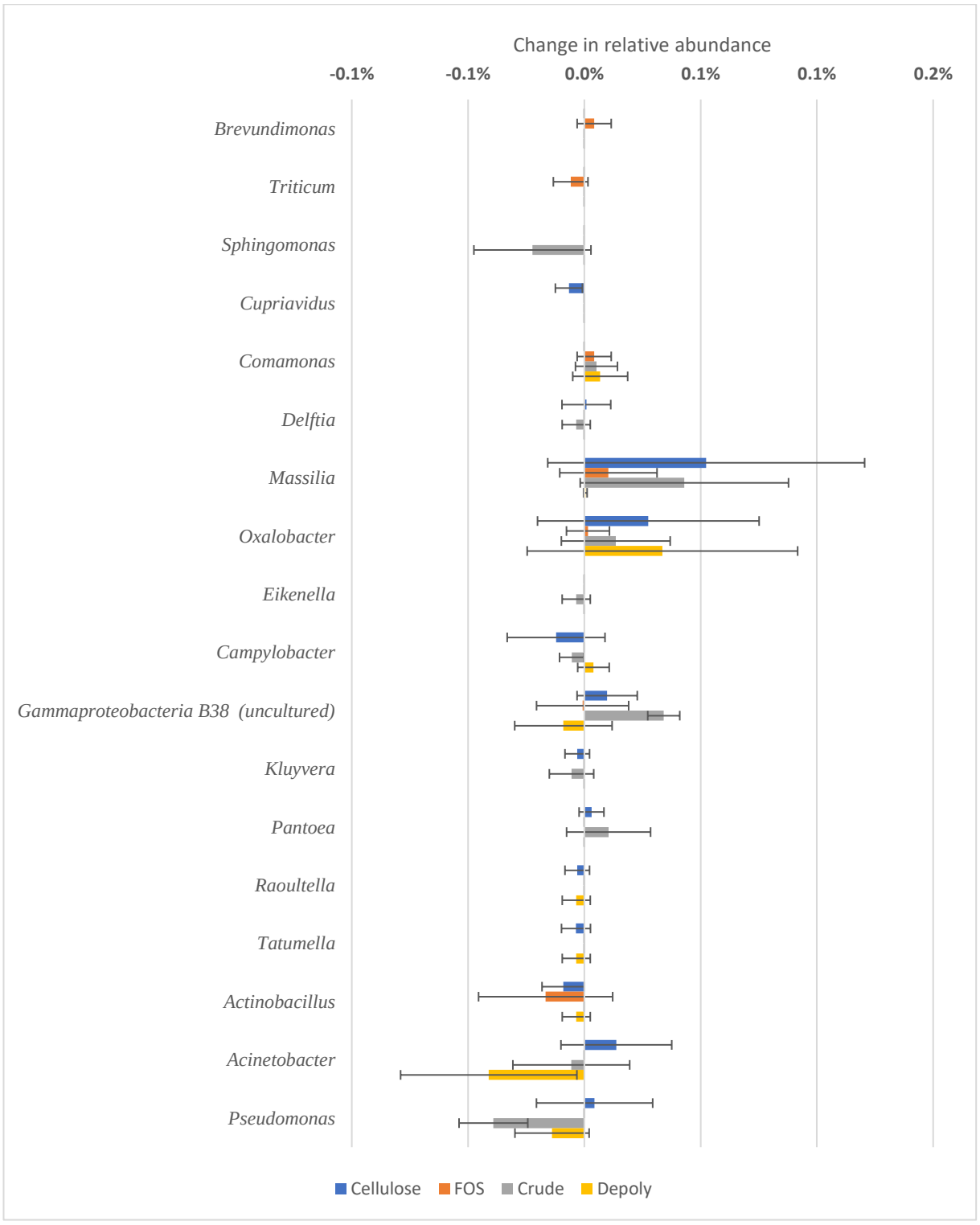

% change
-500
1500
3500
5500
Actinomycetaceae
Bifidobacteriaceae
Coriobacteriaceae
Corynebacteriaceae
Corynebacteriales (uncultured)
Dermatophilaceae
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Bacteroidales S24-7
Bacteroidales (uncultured)
Bacteroidetes VC2.1 Bac22 (uncultured)
4C0d-2 (uncultured)
Fibrobacteraceae (uncultured)
Bacilli 4-15 (uncultured)
Bacillales Family XI Incertae Sedis
Planococcaceae
Lactobacillaceae
Streptococcaceae
Christensenellaceae
Clostridiaceae
Clostridiales Family XIII Incertae Sedis
Clostridiales Family XI Incertae Sedis
Lachnospiraceae
Peptococcaceae
Peptostreptococcaceae
Ruminococcaceae
Veillonellaceae
Clostridiales (uncultured)
Thermoanaerobacterales SRB2
Thermoanaerobacteraceae
Erysipelotrichaceae
Cetobacterium
Victivallaceae
Caulobacteraceae
Brucellaceae
Rhodospirillaceae
Rickettsiales mitochondria
Sphingomonadaceae
Alcaligenaceae
Burkholderiaceae
Comamonadaceae
Oxalobacteraceae
Neisseriaceae
Desulfovibrionaceae
Campylobacteraceae
Gammaproteobacteria B38 (uncultured)
Enterobacteriaceae
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Xanthomonadaceae
Bacteria RF3 (uncultured)
Anaeroplasmataceae
Mollicutes RF9 (uncultured)
Opitutae vadinHA64 (uncultured)
Verrucomicrobiaceae
Other
Cellulose
FOS
Crude
Depoly

Figure 4.10 (a) Increase/decrease in relative abundance of genera in the phylum Proteobacteria. (b) Percentage change in the relative abundance of genera in the phylum Proteobacteria. Values represent the mean (±SE).

(a)




Change in relative abundance
-0.1%
-0.1%
0.0%
0.1%
0.1%
0.2%
Brevundimonas
Triticum
Sphingomonas
Cupriavidus
Comamonas
Delftia
Massilia
Oxalobacter
Eikenella
Campylobacter
Gammaproteobacteria B38 (uncultured)
Kluyvera
Pantoea
Raoultella
Tatumella
Actinobacillus
Acinetobacter
Pseudomonas
Cellulose
FOS
Crude
Depoly

(b)

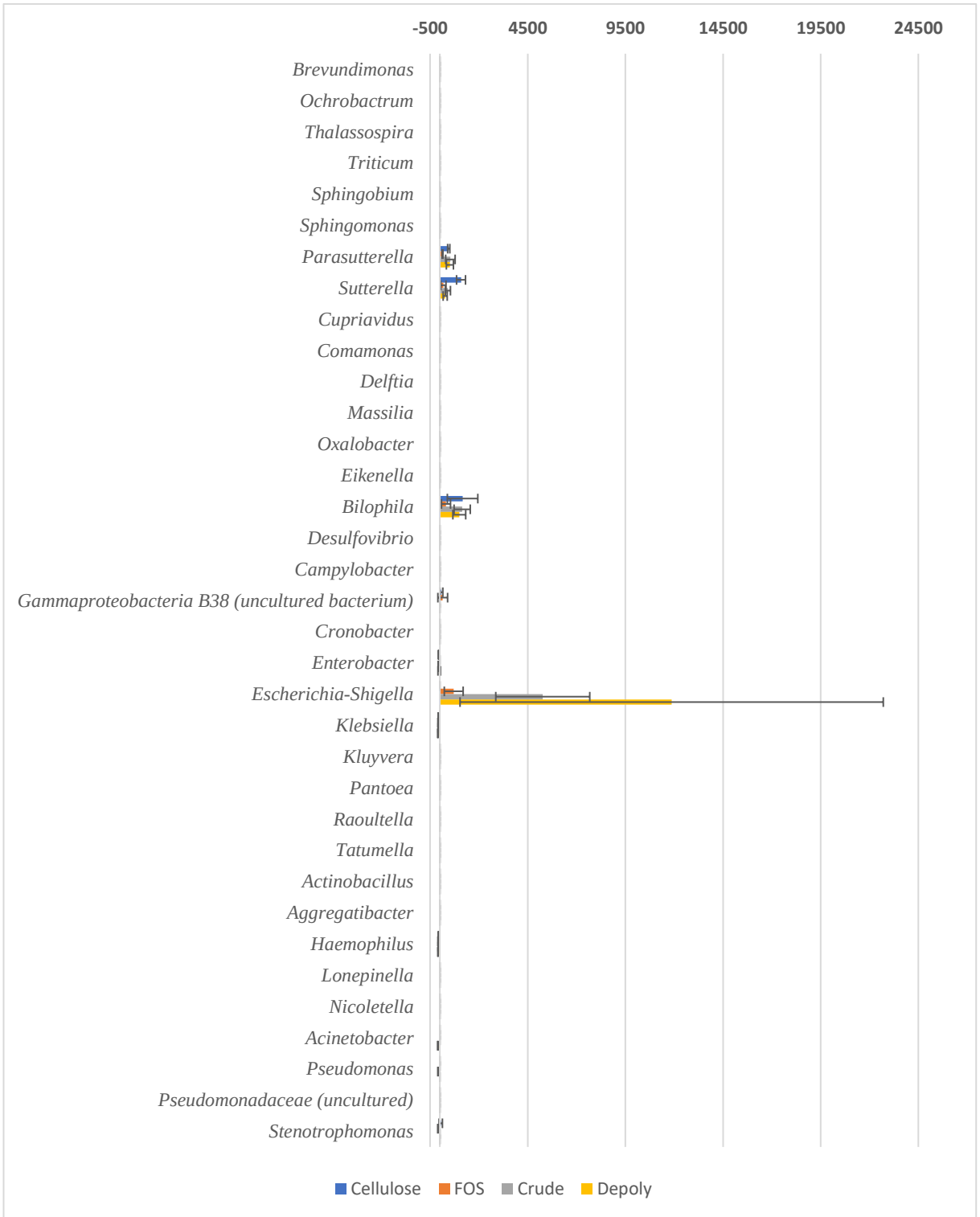
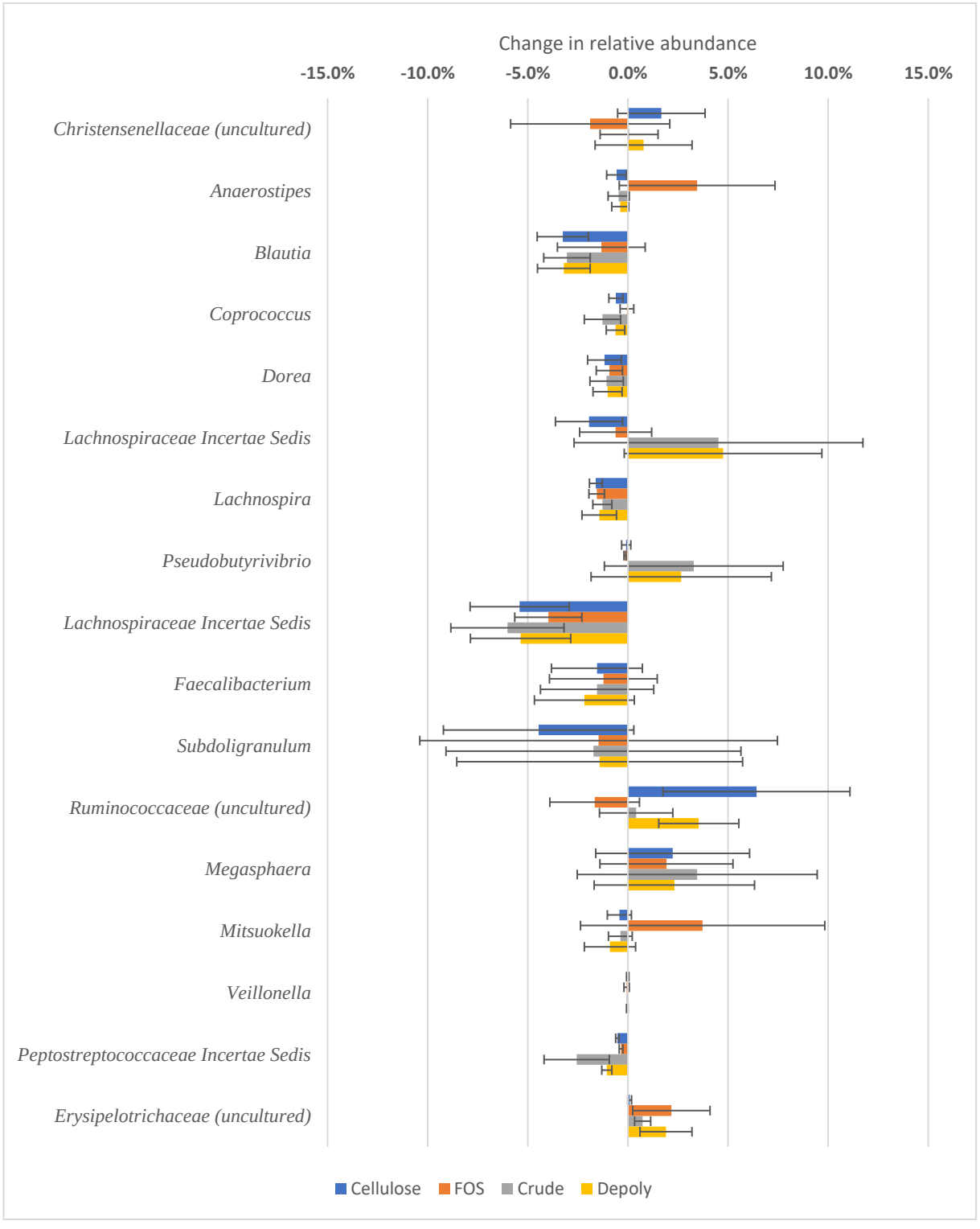
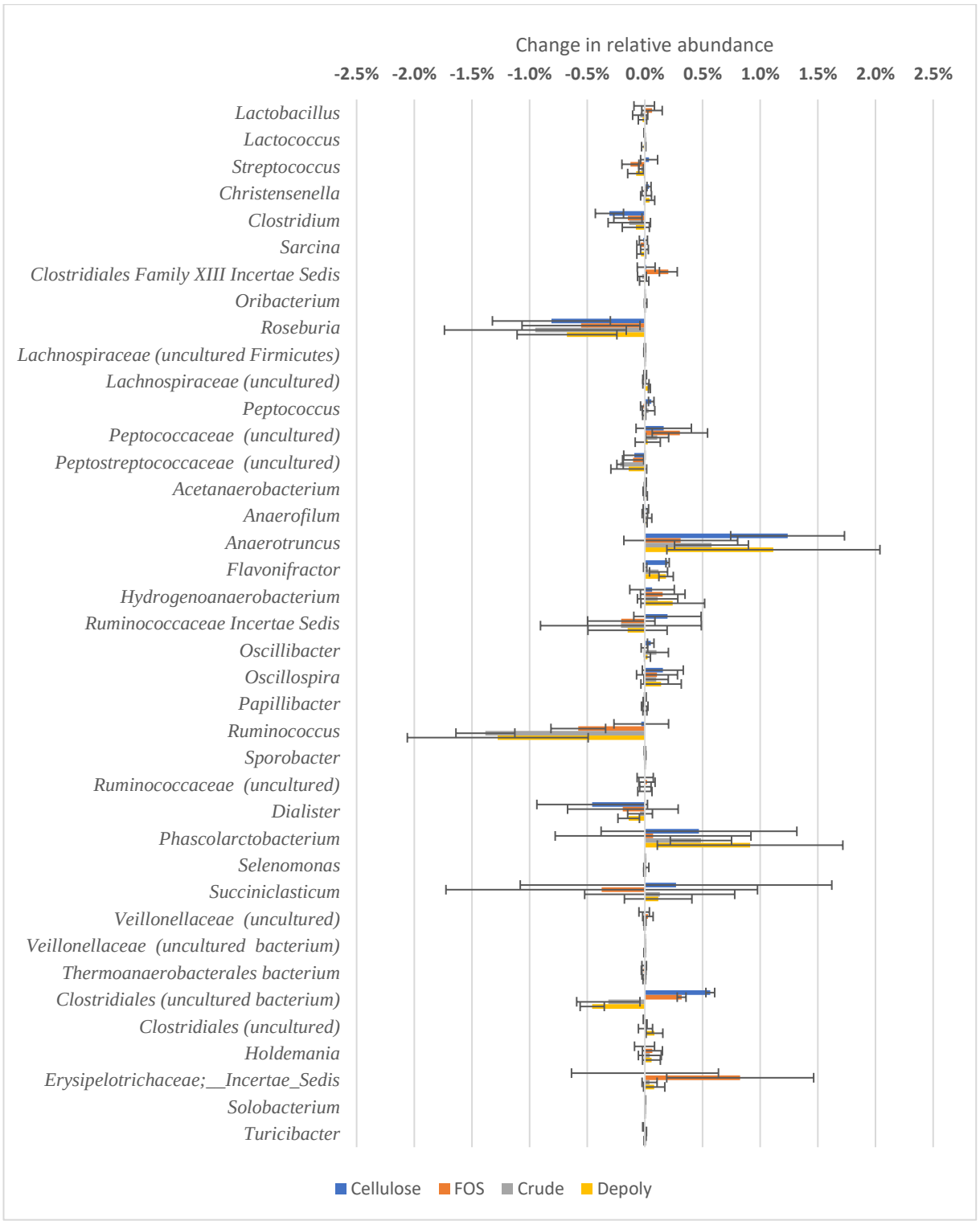

-500
4500
9500
14500
19500
24500
Brevundimonas
Ochrobactrum
Thalassospira
Triticum
Sphingobium
Sphingomonas
Parasutterella
Sutterella
Cupriavidus
Comamonas
Delftia
Massilia
Oxalobacter
Eikenella
Bilophila
Desulfovibrio
Campylobacter
Gammaproteobacteria B38 (uncultured bacterium)
Cronobacter
Enterobacter
Escherichia-Shigella
Klebsiella
Kluyvera
Pantoea
Raoultella
Tatumella
Actinobacillus
Aggregatibacter
Haemophilus
Lonepinella
Nicoletella
Acinetobacter
Pseudomonas
Pseudomonadaceae (uncultured)
Stenotrophomonas
Cellulose
FOS
Crude
Depoly

Figure 4.11 (a) Increase/decrease in relative abundance of genera in the phylum Firmicutes. (b) Percentage change in the relative abundance of genera in the phylum Firmicutes. Data represent the mean (±SE).

(a)




Change in relative abundance
-2.5% -2.0% -1.5% -1.0% -0.5% 0.0% 0.5% 1.0% 1.5% 2.0% 2.5%
Lactobacillus
Lactococcus
Streptococcus
Christensenella
Clostridium
Sarcina
Clostridiales Family XIII Incertae Sedis
Oribacterium
Roseburia
Lachnospiraceae (uncultured Firmicutes)
Lachnospiraceae (uncultured)
Peptococcus
Peptococcaceae (uncultured)
Peptostreptococcaceae (uncultured)
Acetanaerobacterium
Anaerofilum
Anaerotruncus
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Oscillospira
Papillibacter
Ruminococcus
Sporobacter
Ruminococcaceae (uncultured)
Dialister
Phascolarctobacterium
Selenomonas
Succiniclasticum
Veillonellaceae (uncultured)
Veillonellaceae (uncultured bacterium)
Thermoanaerobacterales bacterium
Clostridiales (uncultured bacterium)
Clostridiales (uncultured)
Holdemania
Erysipelotrichaceae;__Incertae_Sedis
Solobacterium
Turicibacter
Cellulose
FOS
Crude
Depoly

(b)

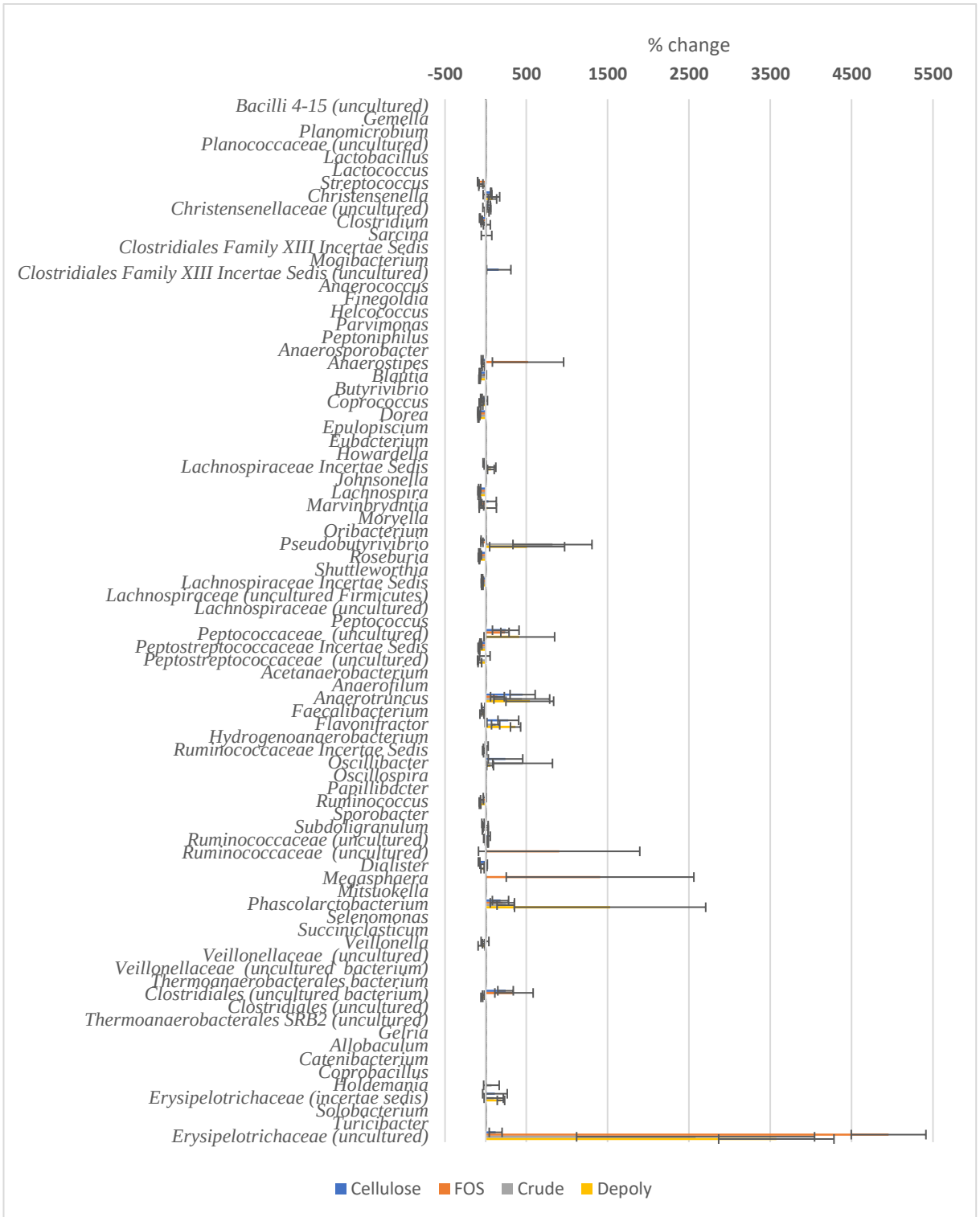
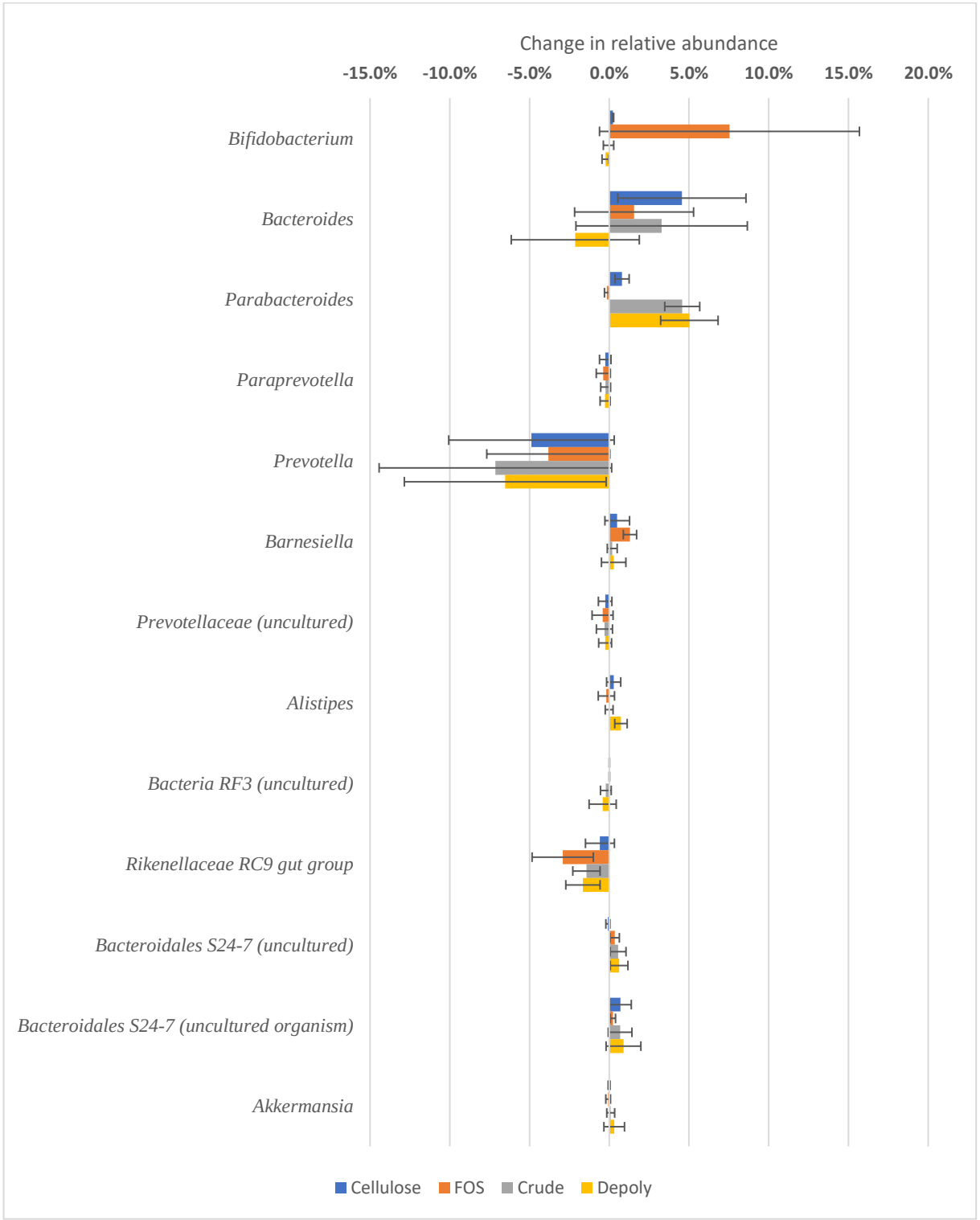
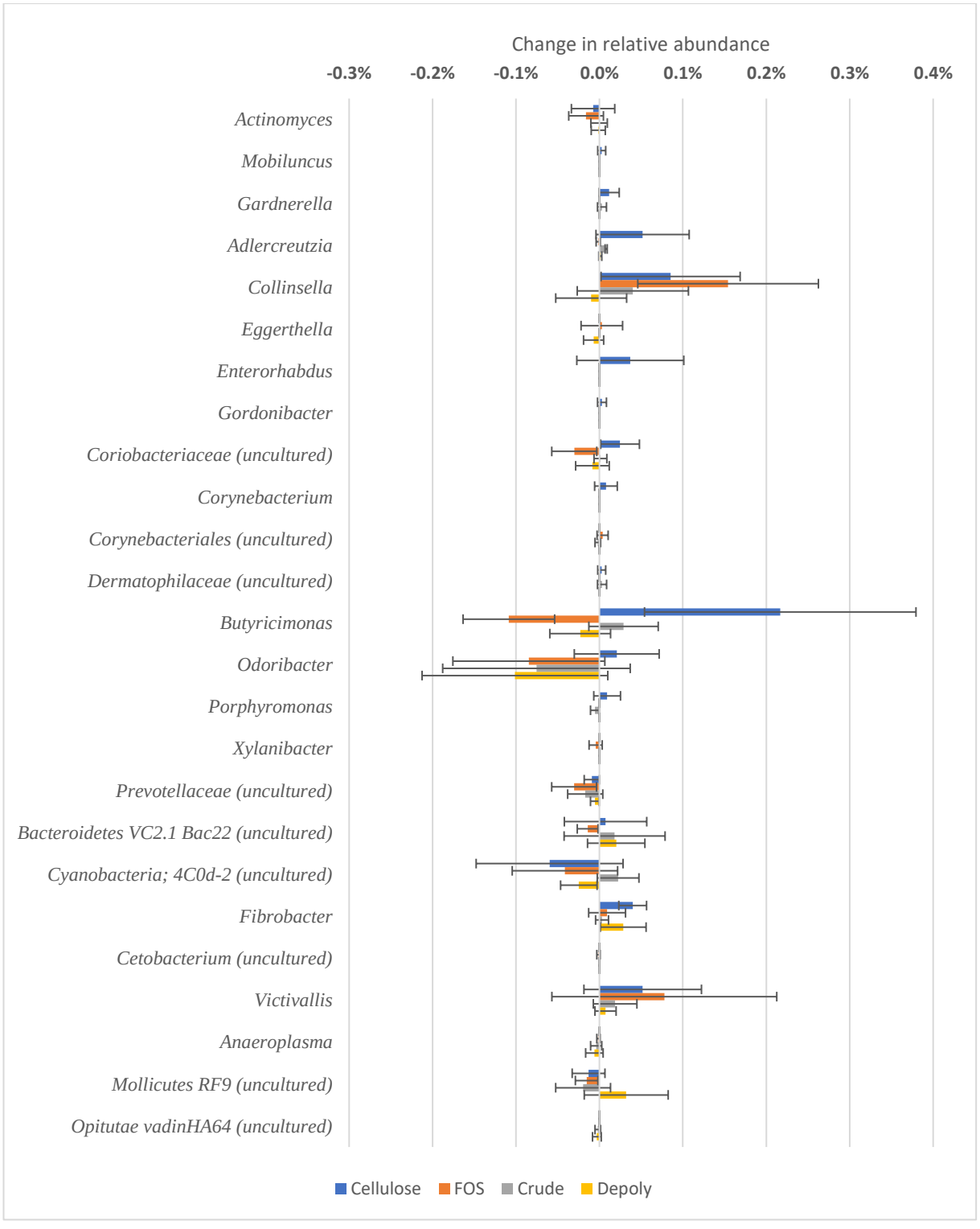

% change
-500
500
1500
2500
3500
4500
5500
Bacilli 4-15 (uncultured)
Gemella
Planomicrobium
Planococcaceae (uncultured)
Lactobacillus
Lactococcus
Streptococcus
Christensenella
Christensenellaceae (uncultured)
Clostridium
Sarcina
Clostridiales Family XIII Incertae Sedis
Mogibacterium
Clostridiales Family XIII Incertae Sedis (uncultured)
Anaerococcus
Finegoldia
Helcococcus
Parvimonas
Peptoniphilus
Anaerosporobacter
Anaerostipes
Blautia
Butyrivibrio
Coprococcus
Dorea
Epulopiscium
Eubacterium
Howardella
Lachnospiraceae Incertae Sedis
Johnsonella
Lachnospira
Marvinbryantia
Moryella
Oribacterium
Pseudobutyrivibrio
Roseburia
Shuttleworthia
Lachnospiraceae Incertae Sedis
Lachnospiraceae (uncultured Firmicutes)
Lachnospiraceae (uncultured)
Peptococcus
Peptococcaceae (uncultured)
Peptostreptococcaceae Incertae Sedis
Peptostreptococcaceae (uncultured)
Acetanaerobacterium
Anaerofilum
Anaerotruncus
Faecalibacterium
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Oscillospira
Papillibacter
Ruminococcus
Sporobacter
Subdoligranulum
Ruminococcaceae (uncultured)
Ruminococcaceae (uncultured)
Dialister
Megasphaera
Mitsuokella
Phascolarctobacterium
Selenomonas
Succiniclasticum
Veillonella
Veillonellaceae (uncultured)
Veillonellaceae (uncultured bacterium)
Thermoanaerobacterales bacterium
Clostridiales (uncultured bacterium)
Clostridiales (uncultured)
Thermoanaerobacterales SRB2 (uncultured)
Gelria
Allobaculum
Catenibacterium
Coprobacillus
Holdemania
Erysipelotrichaceae (incertae sedis)
Solobacterium
Turicibacter
Erysipelotrichaceae (uncultured)
Cellulose
FOS
Crude
Depoly

Figure 4.12 (a) Increase/decrease in relative abundance of genera in the phyla Actinobacteria, Fibroacteres, Lentisphaerae, Tenericutes, and Verrocomicrobia. (b) Percentage change in the relative abundance of genera in the phyla Actinobacteria, Fibroacteres, Lentisphaerae, Proteobacteria, Tenericutes, and Verrocomicrobia. Data represent the mean (±SE).

(a)



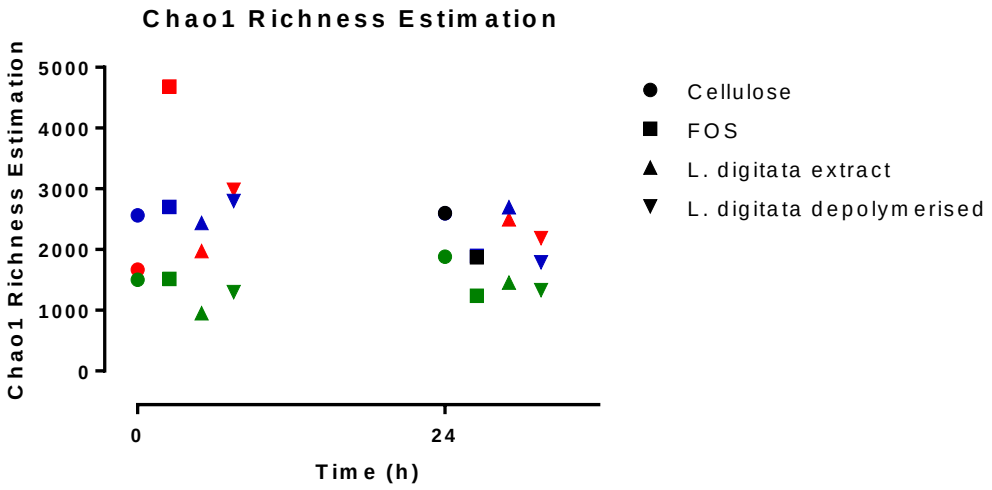

Change in relative abundance
-0.3%
-0.2%
-0.1%
0.0%
0.1%
0.2%
0.3%
0.4%
Actinomyces
Mobiluncus
Gardnerella
Adlercreutzia
Collinsella
Eggerthella
Enterorhabdus
Gordonibacter
Coriobacteriaceae (uncultured)
Corynebacterium
Corynebacteriales (uncultured)
Dermatophilaceae (uncultured)
Butyricimonas
Odoribacter
Porphyromonas
Xylanibacter
Prevotellaceae (uncultured)
Bacteroidetes VC2.1 Bac22 (uncultured)
Cyanobacteria; 4C0d-2 (uncultured)
Fibrobacter
Cetobacterium (uncultured)
Victivallis
Anaeroplasma
Mollicutes RF9 (uncultured)
Opitutae vadinHA64 (uncultured)
Cellulose
FOS
Crude
Depoly

(b)

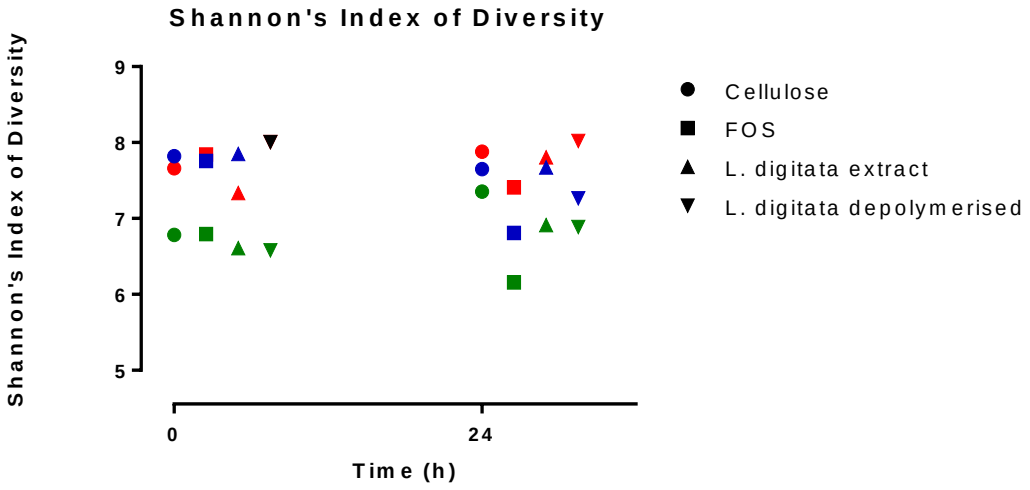

% change
-500
500
1500
2500
3500
4500
5500
6500
7500
Actinomyces
Mobiluncus
Bifidobacterium
Gardnerella
Adlercreutzia
Collinsella
Eggerthella
Enterorhabdus
Gordonibacter
Coriobacteriaceae (uncultured)
Corynebacterium
Corynebacteriales (uncultured)
Dermatophilaceae (uncultured)
Bacteroides
Barnesiella
Butyricimonas
Odoribacter
Parabacteroides
Porphyromonas
Paraprevotella
Prevotella
Xylanibacter
Prevotellaceae (uncultured)
Alistipes
Rikenellaceae RC9 gut group
Bacteroidales S24-7 (uncultured)
Bacteroidales S24-7 (uncultured organism)
Prevotellaceae (uncultured)
Bacteroidetes VC2.1 Bac22 (uncultured)
Cyanobacteria; 4C0d-2 (uncultured)
Fibrobacter
Victivallis
Bacteria RF3 (uncultured)
Anaeroplasma
Mollicutes RF9 (uncultured)
Opitutae vadinHA64 (uncultured)
Akkermansia
Other
Cellulose
FOS
Crude
Depoly

Figure 4.13 The effect of fermentation of *L. digitata* extracts on alpha diversity was measured by several different methods, (a) Chao1 Richness Estimation, (b) Shannon's index of Diversity, (c) Simpson Index of Diversity, (d) Observed species, and (e) PD Whole Tree.

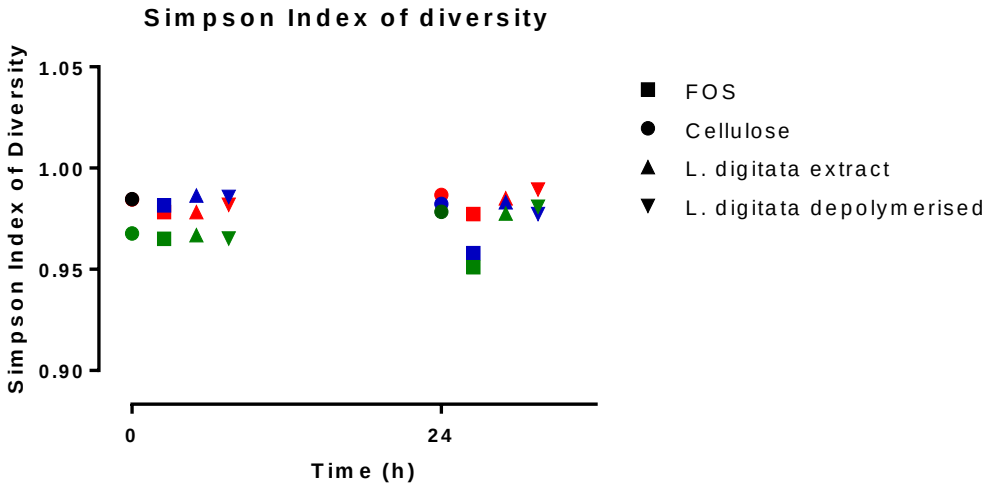
(a)



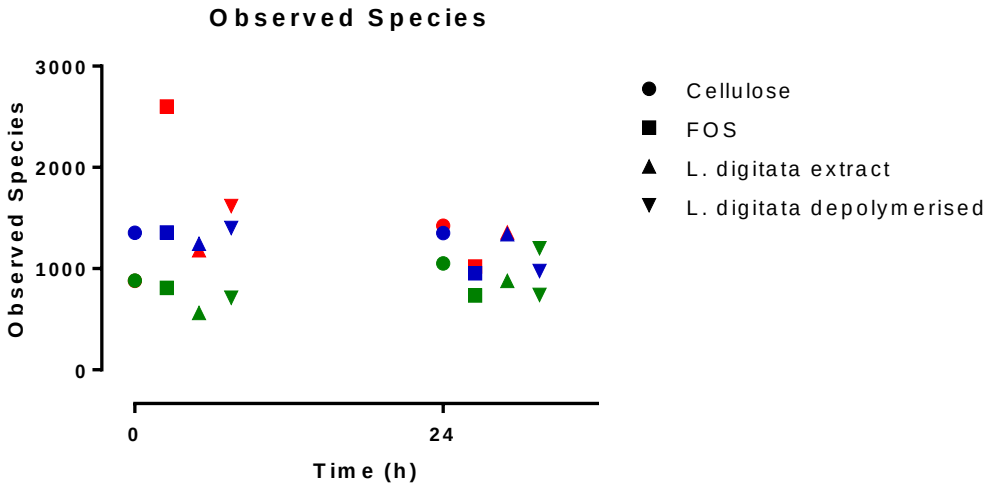
(b)



(c)


Simpson Index of diversity
Simpson Index of Diversity
1.05
1.00
0.95
0.90
0
24
Time (h)
FOS
Cellulose
L. digitata extract
L. digitata depolymerised

(d)


Observed Species
Observed Species
3000
2000
1000
0
0
24
Time (h)
Cellulose
FOS
L. digitata extract
L. digitata depolymerised

(e)

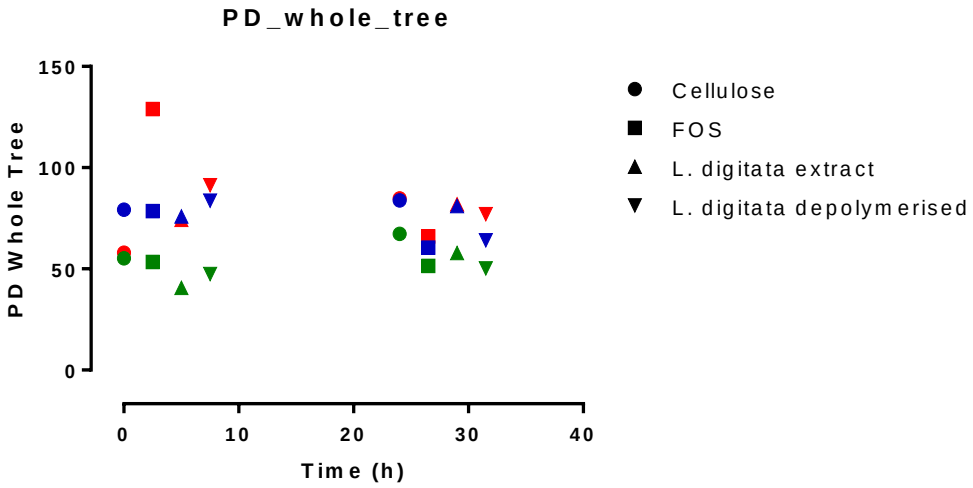
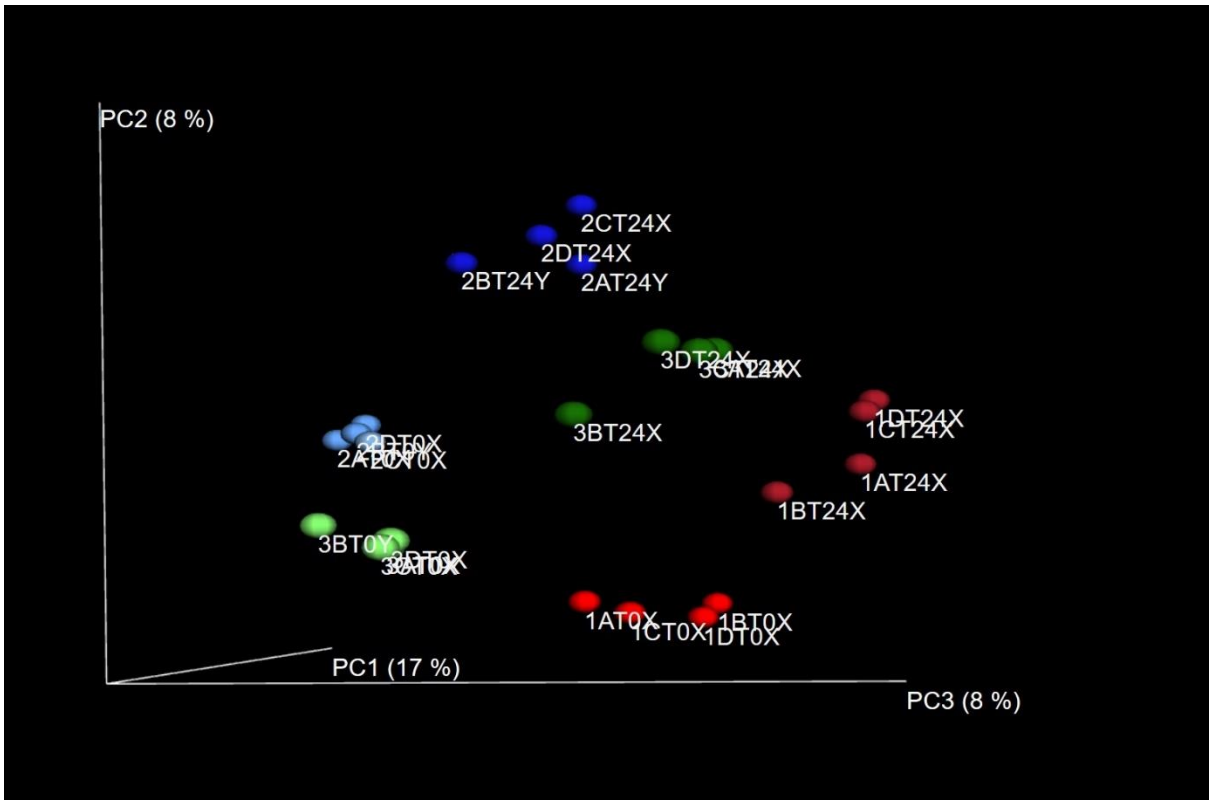

PD_whole_tree
PD Whole Tree
150
100
50
0
0
10
20
30
40
Time (h)
Cellulose
FOS
L. digitata extract
L. digitata depolymerised

Figure 4.14 Beta diversity. Principle coordinate analysis of unweighted Unifrac reveals separation by fermentation run. Red (light) - run 1 0 h, Blue (light) - Run 2 0 h, Green (light) R3 0 h, Red (dark) - Run 1 24 h, Blue (dark) - Run 2 24 h, Green (dark) - R3 24 h.



Supplementary Table 4.1 An overview of the major biologically important SCFA and their physiological effects.

SCFA	Producer bacteria	Physiological effects
Acetate	*Bifidobacterium adolescentis,* *Lactobacillus spp.,* *Bacteroides thetaiotaomicron*	• Energy • Induction of cholesterol biosynthesis
Propionate	*Roseburia insulinovorans* *Veillonella* spp. *Ruminococcus obeum* *Bacteroides* spp. *Dialister* spp. *Phascolarctobacterium* spp.	• Energy in normal cells • Gluconeogenesis • Inhibition of cholesterol biosynthesis • HDACs inhibition
Butyrate	*Roseburia spp.* *Eubacterium rectale* *Eubacterium hallii* *Faecalibacterium prausnitzii* *Anaerostipes caccae* *Coprococcus eutactus*	• Energy in normal cells, • Anti-inflammatory induction of immunosuppressive cytokines, • Induction of immunosuppressive GLP-2 • HDACs inhibition in tumour cells • Apoptosis induction in tumour cells • Overexpression of detoxifying enzymes • Enhancement of mucosal barrier function

Chapter 5

A study of the prebiotic potential of the economically important seaweed *Chondrus crispus* using an *ex vivo* faecal fermentation approach.

5.1 Abstract.

Chondrus crispus is an economically and ecologically important red seaweed with a long tradition of usage on the island of Ireland. Historically known as Irish moss, *C. crispus* was extensively used in cooking as well as in herbal medicines. As a member of the rhodophyta grouping of seaweeds, *C. crispus* contains large quantities of complex polysaccharides such as agarans, xylans and carrageenans which resist degradation in the gastrointestinal tract and reach the colon intact. As such they can be considered a source of dietary fibre and a possible new source of prebiotics. Here, a depolymerised polysaccharide-rich extract from *C. crispus* was investigated for prebiotic potential using *in vitro* batch faecal fermentations. Some significant increases ($p<0.05$) were recorded in the production of total short-chain fatty acids, particularly the biologically important SCFAs propionate. However, there was no significant alteration in the molar ratio of SCFA production or impact on the production of butyrate. High-throughput DNA sequencing revealed that there was no notable impact on the relative abundance of the major probiotic genera, the bifidobacteria and lactobacilli. The results of this study revealed that fermentation of depolymerised polysaccharides from *C. crispus* have only a minimal stimulatory effect on the *ex vivo* microbial population and would not be considered prebiotic by the current definition of the term.

5.2 Introduction

Seaweeds are a very visible part of the global marine landscape. They are a commercially valuable resource that are used for food, fodder, agricultural fertilizers and in the pharmaceutical industry [1]. Worldwide, 96% of the harvested seaweeds are produced by aquaculture which in 2013 had an economic value of $6.4 billion. In 2014 the combined annual seaweed harvest from wild and cultivated crops was 28.4 million tonnes, an increase of 43% since 2010. Some 75% of the global seaweed harvest in 2014 was used for food, with 40% alone being used in traditional Japanese cuisine. The production of hydrocolloids (polysaccharides) such as carrageenan, agar and alginate, accounted for 13% of the global harvest with the remaining 12% used for agriculture [2]. There are essentially two ways of obtaining a economical supply of seaweed material-harvesting natural seaweed stocks or through cultivation. Numerous options for the mechanical harvesting of stocks exist. However, serious concerns exist regarding environmental damage. At present, the mechanical harvesting of seaweeds is banned in the UK and Ireland and all harvesting is carried out by hand [3]. The use of seaweed in biorefinery started in the 17th century with the production of industrial soda and alginate in France and Ireland, and then later in 1893, by Edward Curtis, for iodine extraction with light provided by seaweed derived biogas. During the First World War, because of a shortage of potash, [4] kelps (brown seaweeds) were used to produce the acetone needed for cordite-based guns and artillery shells. The minerals from the seaweed were then recycled and used as fertilizer. Seaweeds contain high levels of proteins and a variety of different polysaccharide molecules. There are thousands of as-yet-undiscovered species of seaweed which promises new unique varieties of polysaccharides with different biochemical properties. As the components of seaweed undergo seasonal fluctuations, the maximum levels of components seldom coincide with each other [5]. Seasonal variation in the amount of carbohydrates in seaweeds is quite large and has necessitated the seasonal harvesting of seaweeds and the development of proper storage technologies, as seaweeds are known to decompose very quickly. A variation of between 5 and 32% for mannitol, for example, in *Laminaria digitata* over a whole year has been reported. [6].

The seaweed *C. crispus* is historically important on the island of Ireland. Alternatively known as Irish Moss, it has been used since the 19th century in folk medicine to treat respiratory ailments such as colds, influenza and tuberculosis. During the great Irish famine of 1845 – 1849 the usage of *C. crispus* was brought to the North-Eastern corner of the United States of America by an Irish emigrant population fleeing starvation. Small quantities are still collected in Ireland for use in cooking and in health drinks [7, 8]. *C. crispus* is a member of the rhodophyta (red seaweeds) which, along with the phaeophyta (brown seaweeds) and the chlorophyta (green seaweeds), is one of the main divisions of macroalgae based on colouration of the thallus [9]. The colouring of the red seaweeds comes from the dominance of the two pigments phycoerythrin and phycothcyanin that mask the effect of other pigments [10]. Red seaweeds in general are particularly rich in bioactive proteins, carbohydrates (including sulphated polysaccharides, agarans, xylans and carrageenans), vitamins and minerals, and pigments [11]. *C. crispus* contains good quantities of micro and macro elements, fatty acids (> 80% unsaturated fatty acids) such as palmitic, palmitoleic, oleic, arachidonic and eicosapentanoic acids, and sterols which are potentially be beneficial to human health [11]. *C. crispus* is also an economically and ecologically important seaweed species [12]. It grows plentifully in the intertidal zone of the Northern to mid-Atlantic region and has been harvested for decades for carrageenans (cell wall polysaccharides) which are used extensively as a thickener in the food industry [13]. The traditional harvesting of *C. crispus* was by local fisherman or farmers by means of drag rakes, hand rakes or by the collection of plants tossed on the shore. The plant was then dried, baled and exported for the extraction of the carrageenans [14]. The carrageenans are a family of high molecular weight (200-800 kDa) sulphated polysaccharides found in red seaweeds and are of a great industrial importance [15, 16]. Sulphated polysaccharides can interact with matrix and cellular proteins owning to their chemical structures which are rich in polyanions [17]. Carrageenans consist of repeated alternating units of α - (1, 3) - galactose and β - (1, 4, 3, 6) - anhydro - D - galactose. Carrageenans are classified into three industrially relevant types, kappa (κ) , lambda (λ) - and iota (ι) – carrageenans (Supplementary Fig. 5.1), which differ in the amount and position of their ester sulphate substitutes and their (3, 6) - anhydrogalactose content [18]. Variations in these components influence hydration, gel strength and texture, melting and setting temperature etc [19].

κ- and ι-carrageenan contain the (3, 6) - anhydro-galactose unit and are the gel forming types of carrageenan [15]. Typically, κ-carrageenan forms hard, strong and brittle gels, while ι-carrageenan forms elastic, soft and weak gels [20]. λ-carrageenan is a very high-sulphated type of carrageenan that lacks the (3, 6) - anhydro-galactose unit and does not gel. It is mainly used for its ability to provide a mouth feeling and a creamy impact to dairy products [16]. Carrageenans have shown several potential pharmaceutical applications including anti-coagulant, anti-cancer, anti-hyperlipidemic, and immune-modulatory activities. Along with *C. crispus* other major raw sources for carrageenan extraction include *Gigartina stella*, *Eucheuma* spp., *Iridaea* spp., and *Kappaphycus* spp [21]. Seaweed derived hydrocolloids, as of 2015, have a global value of approximately US$ 1.1 billion, which is projected to increase in the future. While agars have the highest retail price per kg (18 US$/kg), carrageenans currently have the highest commercial production (60,000 ton/year) and have the highest total value of US$ 626 million per year [22]. Originally, carrageenans were first isolated in 1862 by a British pharmacist named Stanford who extracted them from Irish Moss [23]. The modern carrageenan industry dates to the 1940s when they were used in the dairy industry as the perfect stabilizer for the suspension of cocoa in chocolate milk. They are also widely used in ice cream, paints, water gels and pharmaceuticals. Carrageenans are extracted from seaweeds by two distinct processes. In the refined process, the carrageenan is dissolved completely out of the cellulose matrix of the seaweed leaving a very clean and clear product. The semi refined process leaves the cellulose intact, thus enhancing the gel strength of the carrageenan within the seaweed itself. The final semi-refined product has excellent gelling and binding properties at a reduced cost, but the gel formed is less clear than the refined version [20].

Complex seaweed polysaccharides may also give rise to a new and novel source of prebiotic compounds. [24]. A prebiotic is “a selectively fermented food ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” [25]. The rising concern among individuals, regarding maintaining good levels of overall health and with a growing emphasis on digestive health, is driving growth in the prebiotic market. High demands from the food and beverage sectors are also boosting the industry. The global prebiotics market is predicted to reach US$ 15.90 billion by 2019

according to research by Transparency Market Research [26]. The inclusion of prebiotic food ingredients in the diet may facilitate changes in the GIT environment that favour the proliferation of probiotic bacteria such as *Bifidobacterium* and *Lactobacillus*. In general, the aim of prebiotic administration is to increase the saccharolytic activity of the colonic bacteria and promote carbohydrate fermentation, the end products of which (SCFAs) have beneficial effects. Concomitantly, the fermentation of undigested proteins, which results in the production of potentially toxic metabolites, would be expected to be reduced owning to a change in the fermentation profile of the GIT [27]. The main SCFAs produced by the microbiota are acetic acid (acetate), propionic acid (propionate) and N-butyric acid (butyrate). SCFAs are believed to provide an energy source for colonic cells and act as anti-proliferation agents [28]. The aim of this investigation was to assess the prebiotic potential of the economically important red seaweed *C. crispus*.

Here, a polysaccharide-rich extract (the Cc extract) was prepared from the commercially valuable red seaweed *C. crispus.* Prebiotic potential was measured by subjecting the Cc extract to an *ex vivo* faecal batch fermentation. Samples of fermentate were taken at various time points for short-chain fatty acid production, DNA sequencing, enumeration of *Bifidobacterium* and *Lactobacillus* and the production of hydrogen sulphide.

5.3 Material and methods

5.3.1 Materials

All laboratory materials were obtained from Sigma-Aldrich, Dublin, Ireland unless otherwise stated. All plastic consumables were obtained from Sarstedt Ltd, Wexford, Ireland.

5.3.2 Seaweed origin.

The seaweed used in this study was the red seaweed species *Chondrus crispus* ISGC0439. This specimen of *C. crispus* was collected from the Galway bay region of Ireland.

5.3.3 Preparation of depolymerized *C. crispus* polysaccharide rich extract.

The *C. crispus* (ISCG0439) sample was washed with cold water and subsequently stored at - 20 °C until required for extraction. The extraction method chosen for this work was a hot-acid extraction using hydrochloric acid (37% v/v). Prior to the primary extraction, the *C. crispus* sample was removed from cold storage, blended to a fine powder and added to a large reaction vessel along with deionised water in a ratio of 1:10 (w/v) seaweed/deionised water. HCl was added (8.25 ml/L) to the reaction vessel to give a final concentration of 0.1 M. The reaction vessel was then placed in an orbital shaker and allowed to shake at 75 rpm for 3 h at a temperature of 70 °C, after which the reaction vessel was removed from the shaker and allowed to cool. The contents of the vessel were filtered through a muslin bag with the filtrate being retained and transferred to a separate, clean storage vessel. The remaining seaweed mass was placed back into the original reaction vessel with fresh reagents and treated as previously described. The combined filtrates were neutralised using 1M NaOH (pH 6-8) prior to storage and were subsequently centrifuged at 5000 g for 5 min to remove insoluble particulate matter before being blast-frozen and freeze-dried.

5.3.4 Post primary extraction processing of the *C. crispus* extract.

The evident gelling properties of the *C. crispus* polysaccharides necessitated that the primary extract was subjected to a depolymerisation step. The extract was dissolved in 0.04% $FeSO_4$ (1:5 w/v) solution to which hydrogen peroxide (30% puriss grade) was added (1:40 v/v) using a ratio of extract to hydrogen peroxide of 1:25. The mixture was allowed to shake at 150 rpm in a water bath at 80 °C for 15 min. Following the depolymerisation process, the mixture was blast-frozen and freeze-dried prior to storage. Ethanol precipitation was employed to separate the seaweed polysaccharides from the non-polysaccharide content and to remove impurities. The crude extract powder was resuspended in minimal deionised water and reacted with 100% ethanol in a ratio of seaweed: ethanol of 1:5. The resulting mixture was centrifuged for 5 min at 5000 g. The collected pellet was resuspended in fresh deionised water and blast-frozen and freeze-dried prior to storage. Size exclusion dialysis with a molecular cut off point of 1 kDa was employed to remove simple sugars from the extract. The freeze-dried powder was resuspended in minimum deionized water prior to dialysis. The dialysis tubing was prepared by cutting it into strips of appropriate length (~15cm) and rinsing each strip gently in deionised water to remove traces of the sodium azide storage solution. Each strip of dialysis tubing was filled with *C. crispus* extract and sealed using plastic clips. The tubing was carefully positioned in a washed container filled with deionised water, covered with tinfoil and placed in an orbital shaker and allowed to shake at 40 rpm at 25 °C. Each container had its water replaced every morning with fresh deionized water. On the third day, the dialysis, the tubes were removed from their respective containers and opened. Following dialysis, the collected dialysate underwent a simulated gastric digest. First, α-amylase (200 U) was prepared in 6.25 ml filter sterilized $CaCl_2$ (1mM, pH7) and added to the seaweed mix, which was allowed to shake for 30 min at 37 °C. The pH was adjusted to pH 2 with HCl. Pepsin (2.7 g) dissolved in 125 ml 0.1M HCl was added to the seaweed mixture and incubated at the same conditions as before for 2 h. The pH was adjusted to pH 7 with NaOH. Bile (3.5 g) and pancreatin (560 mg) were dissolved in 0.5 M NaHCO3 and added. The seaweed mixture was set shaking for another 3 hours. To produce the final extract, the *C. crispus* digest underwent a further 1 kDa size-exclusion dialysis to remove breakdown components from the simulated digest.

5.3.5 Preparation of the cellulose negative control.

The cellulose control for the study was processed in a similar manner to the final *C. crispus* (Cc) extract. Firstly, the cellulose was subjected to a simulated gastric digestion followed by size exclusion dialysis as previously outlined. Finally, the cellulose (post dialysis) was blast-frozen and freeze-dried to produce the final cellulose control.

5.3.6 Carbohydrate analysis.

The total concentration of carbohydrates in the Cc extract, FOS and cellulose control was determined by a resorcinol sulphuric acid method [29] with modifications. Briefly, a 75% H_2SO_4 solution, 6 mg/ml resorcinol stock solution, and a glucose control solution of 200 µg/ml were prepared using fresh deionised water. Each sample was prepared at a concentration of 1 mg/ml. To each microtube, 200 µl sample, 200 µl resorcinol solution, and 1 ml of 75% sulphuric were added. Each microtube was covered in tinfoil and vortexed for 30 sec. The samples were then incubated at 90 °C for 30 min. after which they were placed at room temperature and allowed to cool. Readings at an optical density of 430_{nm} and 480_{nm} were taken respectively and an average value of total carbohydrates was obtained.

To evaluate the polysaccharide content of the Cc extract, 2M trifluoroacetic acid (TFA) was used to degrade the extract to its component monosaccharides. The Cc extract prepared at a concentration of 1 mg/ml in the TFA and was reacted at 80 °C for 90 minutes with constant stirring. The concentration of glucose and galactose in the degraded Cc extract were then determined using a HPLC method with an Aminex HPX 87C fixed ion resin column. The column was set at 60 °C and the eluent (0.009N H_2SO_4) was run at 0.5 ml/min. The sugars were detected using a refractive index detector attached to the system. The Cc extract was filtered through a 0.22 µm microfilter before subjecting to HPLC. Calibration curves for the 3 sugars were generated using different concentrations 10, 20, 50, and 100 ug/ml and gave a linear response [30].

5.3.7 *Ex vivo* faecal fermentation distal colon model.

The fermentation media used in this experiment was prepared according to Fooks et al., [31] with modification. The media consisted of: tryptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), KH_2PO_4 (0.04 g/l), K_2HPO_4 (0.04 g/l), $CaCl_2.6H_20$ (0.04 g/l), $MgSO_4.7H_2O$ (0.01 g/l), sodium bicarbonate (2 g/l), tween 80 (2ml/l), hemin (0.05 g/l), vitamin K1 (10 µl/l), cysteine HCl (1 g/l) and bile salts (0.5 g/l). The medium (800ml) was adjusted to pH 6.8 and autoclaved at 121 °C for 15 min. On the morning of each experiment, 2 g of either control or seaweed extract were dissolved in 160 ml of the fermentation media which in turn were added aseptically to their respective vessel in the MultiFors fermentation system (Infors UK). The system and media were sparged with N_2 for at least 120 min to ensure an oxygen free anaerobic condition was established. Faecal samples were obtained from at least three healthy volunteers aged between 22 and 50 years. Each volunteer had no history of bowl problems and had not taken a course of antibiotics in the previous 6 months. The samples were collected roughly 2 h beforehand and were combined in a sterile filter stomacher bag to form a composite faecal sample. Immediately prior to the inoculation of the fermenters, the composite sample was mixed for 90 sec in the stomacher with an appropriate volume of maximum recovery diluent (MRD) containing 0.05% L-cysteine hydrochloride adjusted to pH 6.5 (which had been boiled and cooled in the anaerobic cabinet post autoclaving) to give a 20% composite faecal solution. Forty millilitres (40 ml) of the composite faecal slurry were introduced into each respective fermentation vessel. The final volume for each vessel was 200 ml. Samples (1ml aliquots) were taken at 0 h, 5 h, 10 h, 24 h, 36 h, and 48 h for total short chain fatty acid analysis, pyrosequencing and hydrogen sulphide production analysis. Plate counts were carried out at 0, 5, 10 and 24 h to enumerate the major probiotic genera, *Bifidobacterium* and *Lactobacillus.* The negative control used for this experiment was cellulose and the positive control used was the known prebiotic fructooligosaccharide (FOS). The faecal fermentation was repeated three times (n = 3) with samples being taken at each time point in at least duplicate.

5.3.7 Analysis of short-chain fatty acid production.

Samples of fermentation media were taken in triplicate at all time points for SCFA determination. Each sample was centrifuged at 12500 g for 15 min with the supernatant being stored at -80 °C prior to further processing. Each sample was later thawed on ice and centrifuged at 12500 g for 5 min and filtered using 0.22 µm filters to remove bacteria and other solids. Samples were diluted 1:5 with Milli-Q water and 1 mM 2-ethylbutyric acid, prepared in formic acid, was added to each sample as an internal control. SCFA analysis was carried out using a Varian CP-3800 GC system with a Flame Ionisation Detector (FID). This was fitted with a Zebron ZB-FFAP capillary column (30 m length x 0.32 mm internal diameter x 0.32 µm film thickness). Helium was used as the carrier gas and had an initial flow rate of 1.3 ml/min. The oven settings were as follows; 1) initial oven temperature was 100 °C - held for 30 sec; 2) temperature raised to 180 °C at 8 °C/min - held for 1 min; 3) temperature increased to 200 °C at 20 °C/min – held at 200 °C for 5 min. The temperatures of the detector and the injection port were set at 250 °C and 240 °C respectively. A calibration curve was produced using different concentrations of a seven SCFA standard mix (10.0 mM, 8.0 mM, 4.0 mM, 2.0 mM, 1.0 mM and 0.5 mM). The sample injection volume was 0.5 µL. Additional vials containing standards were included in each run to maintain calibration and a cleaning injection of 1.2% formic acid was used before each analysis. The peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software. The SCFAs investigated in this study were acetic acid (acetate), propionic acid (propionate), N-butyric acid (butyrate), isobutyric acid (isobutyrate), valeric acid (valerate), isovaleric acid (isovalerate) and hexanoic acid (hexanoate).

5.3.8 Preparation of DNA for high-throughput pyrosequencing.

Fermentation samples (1 ml) were collected at time points 0 h and for pyrosequencing analysis. The MO BIO PowerFecal® DNA Isolation Kit was used to extract total bacterial genomic DNA. Genomic DNA was stored at - 20 °C following extraction.

The microbiota composition of each sample was established by amplicon sequencing of the V4 region using universal 16S rRNA primers predicted to bind to 94.6% of all 16S rRNA genes [32, 33]. A forward primer (5'- AYTGGGYDTAAAGNG) containing a distinct multiple identifier tag (MID) for each sample (Table 3.2) and a combination of 4 reverse primers, R1 (5'- TACNVGGGTATCTAATCC), R2 (5'- CTACDSRGGTMTCTAATC), R3 (5'- TACCAGAGTATCTAATTC) and R4 (5'- TACCRGGGTHTCTAAT.CC) were utilised. All primers were synthesised by Eurofins Genomics, Ebersberg, Germany). PCRs were carried out using an Applied Biosystems® 2720 Thermo cycler. DNA samples were heated to 95 °C for 10 min before all PCRs. The cycling conditions for all PCR reactions were as follows: heated lid 110 °C, 94 °C for 2 min followed by 36 cycles of 94 °C for 1 min, 52° C for 1 min and 72 °C for 1 min. This was followed by a final temperature step of 72 °C for 2 min and a holding step at 4 °C. Each PCR reaction had a final volume of 50 µl comprising 25 µl of BioMix Red (MSC, Ireland), 1 µl forward primer (0.15 µM), 1 µl reverse primer (0.15 µM) (mix of 4), 5 µl template DNA, and 18 µl sterile PCR water. All PCRs were carried out in triplicate. Negative controls were run concurrently for all PCR conditions. PCR products were analysed using agarose gel electrophoresis (1.5% in 1 x TAE buffer). Generated amplicons were cleaned with Agencourt AMPure XP (Beckman Coulter) and quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). Samples were sequenced on a 454 Genome Sequencer FLX platform according to established protocols.

5.3.9 Enumeration of probiotic bacterial populations by plate count method.

Samples of fermentation content were taken at each time point between 0 h and 24 h for bacterial plate counts. The media used for *Bifidobacterium* enumeration was modified MRS agar plates (0.05% cysteine, 200 µl mupirocin). *Lactobacillus* selective agar (LBS; Difco, Becton-Dickson ltd, Dublin, Ireland) plates with glacial acetic acid (1.32 ml/L) were prepared for *Lactobacillus* enumeration. Appropriate serial dilutions of faecal aliquots (1 in 10 dilutions) were carried out using maximum recovery diluent (MRD). Plates were incubated anaerobically at 37 °C for 3-5 days before recovery.

5.3.10 Bioinformatic analysis of sequencing data.

Raw sequences were quality-trimmed using the Qiime Suite of programmes [34]; any reads not meeting the quality criteria (a minimum quality score of 25 and a sequence length <150 bp for 16S amplicon reads) were discarded. OTUs were aligned using PyNAST[35] and taxonomy assigned using BLAST[36] against the SILVA SSURef[37] database release 111. α and β diversity indices and rarefaction curves were generated using Qiime. A phylogenetic tree was calculated using the FastTree [38] software and the resulting principal coordinate analysis was visualised within KiNG.

5.3.11 Statistical analysis.

All results presented as the mean value (± SE). Statistical analysis was carried out using GraphPad Prism version 5.0 for windows. Independent t-test were carried out on parametric data sets to access whether differences between the treatment groups and the cellulose control were significant. Statistical significance was accepted at $P < 0.05$.

5.4 Results

5.4.1 Total carbohydrate concentration and compositional analysis of the Cc extract.

The total concentration of carbohydrates was determined for the Cc extract, FOS and cellulose control using a resorcinol sulphuric acid method with modifications. Carbohydrate concentration is presented in terms of Glucose equivalent (GE) per milligram. Total concentration of carbohydrates for the Cc extract was determined to be 52.2 ± 0.3 GE mg^{-1}. Total concentration of carbohydrates for the cellulose control was 105.7 ± 6.3 GE mg^{-1} and for the FOS was 272.1 ± 4.6 GE mg^{-1}.

The polysaccharide content of the Cc extract was investigated by treatment with TFA to degrade the parent polysaccharides to their base monosaccharide components. Prior to treatment with TFA, the levels of lactose, glucose and galactose in the Cc extract were 0 µg/ml. Following treatment of the Cc extract with TFA, the concentration of galactose was 170.5 µg/ml and the concentration of glucose was 6.2 µg/ml. No lactose was detectable following TFA treatment (Fig. 5.1)

5.4.2 Enumeration of bifidobacteria and lactobacilli by plate count.

Serial dilutions of fermentation samples were carried out at 0 h, 5 h, 10 h, and 24 h to enumerate *Bifidobacterium* and *Lactobacillus* numbers (CFU/ml) (Fig 5.2). Results of the plate counts showed that the Cc extract had no significant impact on the recovery of *Bifidobacterium.* No noteworthy effect on *Bifidobacterium* was observed and numbers of recovered bifidobacteria were reduced at every time point. A significant increase in *Bifidobacterium* numbers was overserved with FOS, in comparison with the cellulose control, at 10 h. There was also a significant increase at 10 h over the Cc extract. The recovery of culturable *Lactobacillus* was not significantly impacted by fermentation of the Cc extract or FOS. A reduction in their numbers was observed at every time point during the Cc extract fermentation. Lactobacilli attained their highest numbers at 5 h for both the cellulose control and FOS. Reduction in *Lactobacillus* numbers was observed for both controls at 10 h and 24 h

5.4.3 Short-chain fatty acid production.

Total SCFA production was measured using a Gas Chromatography – Flame Ion Detector (GC-FID). All reported values are minus the baseline level of SCFAs taken at 0 h and are ± the SEM. The total production of SCFA was significantly increased ($p>0.05$) for the FOS fermentation (+167.8% ± 10.6). A non-significant increase in total SCFA production of 13.7% ± 6.1 was observed for the Cc extract (Fig. 5.3). Total SCFA concentration was significantly higher ($p<0.05$) with the Cc fermentation at 24 h and 36 h respectively, in comparison with cellulose. Fermentation of FOS by the representative microbiota resulted in significant increases ($P>0.05$) in the concentration of acetate, butyrate and propionate as well as significant increases in total SCFA concentration. Total SCFA concentration with the FOS control was significantly higher than that observed with the Cc extract at all time points (Fig. 5.3). Acetate was the major SCFA produced for all fermentations, accounting for 45.0% ± 3.7 (cellulose), 52.0% ± 2.6 (FOS) and 48.6% ± 3.0 (Cc extract) of total SCFA production (Fig. 5.4). Total acetate production was increased during the Cc extract fermentation by 10.5% ± 4.9. Neither acetate concentration or production were significantly affected by supplementation with the Cc extract (Fig. 5.5). The second major SCFA produced was butyrate, which accounted for 22.2% ± 1.0 (cellulose), 23.2% ± 0.2 (FOS) and 19.5% ± 2.2 (Cc extract) for total SCFA production (Fig. 5.4) Total butyrate production was reduced by 1.3% ± 8.3 with the Cc extract fermentation. There was no significant impact on butyrate concentrations or production at any time point during the Cc fermentation (Fig. 5.6). The third major SCFA produced was propionate. Propionate production accounted for 16.8% ± 1.0 (cellulose), 19.4% ± 1.3 (FOS), and 19.7% ± 1.3 (Cc extract) (Fig. 5.4) Total propionate production was increased during the Cc extract fermentation by 21.8% ± 2.9. Propionate concentration was significantly higher than the cellulose control at 24 h ($P=0.008$) and at 36 h ($P=0.011$). Propionate production was not significantly affected during the Cc extract fermentation (Fig. 5.7). There was no significant impact on the BCFA concentration or production with the Cc extract of FOS fermentation (Fig. 5.8)

The molar ratio of acetate production: propionate production: butyrate production after with the Cc extract was approximately 58:24:18. Compared with the molar ratio of the control fermentation which was 57:19:24, there was significant increase in the proportion of propionate produced ($p=0.0230$), with an associated

reduction in the amount of butyrate production. Acetate production was not significantly affected. This ratio for the FOS was also not significantly different from the cellulose control. After 48 h the Cc extract ratio returned to the same level as the cellulose control (Table 5.1)

5.4.4 High throughput DNA sequencing of the 16s rRNA gene variable V4 region.

Taxonomy based analysis of the high throughput sequencing data revealed a baseline (T0) assignment of reads as follows. In the cellulose control community, 54.3% ± 3.8 of reads corresponded with Firmicutes, Bacteroidetes 41.5% ± 3.6, Proteobacteria 3.2% ± 0.8, Actinobacteria 0.67% ± 0.2%, with all other phyla accounting for 0.3% ± 0.32. In the FOS community, 52.9% ± 9.1 of assigned sequence reads corresponded with Firmicutes, Bacteroidetes 43.8% ± 9.1, Proteobacteria 2.8% ± 0.1, Actinobacteria 0.7% ± 0.2, with all other phyla accounting for 0.1% ± 0.1. Finally, the Cc extract community had 50.9% ± 5.8 reads assigned to Firmicutes, Bacteroidetes 36.6% ± 3.7, Proteobacteria 11.4% ± 2.3, Actinobacteria 0.7% ± 0.2, and other phyla 0.1% ± 0.1. In comparison with the cellulose control, a significant ($P>0.05$) decrease of change in relative abundance was observed with the FOS fermentation for the phylum Proteobacteria (Fig 5.9). No significant differences were observed between the cellulose control and the Cc extract fermentation in terms of change in relative abundance (Fig. 5.9)

Analysis of reads assigned at a family level showed that the most dominant families present were *Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Porphyromonadaceae* and *Prevotellaceae.* Fermentation of the Cc extract resulted in a significant ($P<0.05$) reduction in change of relative abundance of *Christensenellaceae* (Firmicutes), in comparison with the cellulose control. For the FOS fermentation, significant increases ($P>0.05$) in relative abundance for the family Erysipelotrichaceae (Fig. 5.10) and significant decreases in relative abundance for uncultured Bacteroidales family S24-7 (Fig. 5.10) and Christensenellaceae (Fig. 5.10). In comparison with the FOS control, there were significant increases ($P>0.05$) with the Cc extract in the relative abundance of uncultured Bacteroidales family *S24-7* (Fig. 5.10), Alcaligenaceae *(*Fig. 5.10), and Pseudomonadaceae *(*Fig. 5.10) and a significant reduction in relative abundance for Erysipelotrichaceae (Fig. 5.10)

At the genus level, the most abundant genera were found to be Bacteroides, an uncultured genus of the family Lachnospiraceae, *Prevotella*, *Faecalibacterium*, Lachnospiraceae incertae sedis, and an uncultured genus of the family Ruminococcaceae. There were no significant increases/decreases observed with the Cc extract. At the genus level with the FOS fermentation, in comparison with the cellulose control, there were significant increases (P>0.05) in relative abundance of *Anaerostipes* (Fig. 5.12), and an uncultured genus of the family Erysipelotrichaceae (Fig. 5.12) with significant decreases in relative abundance being observed for the genera *Odoribacter* (Fig. 5.11) an uncultured member of the family Christensenellaceae (Fig. 5.12), Lachnospiraceae incertae sedis (Fig. 5.12), *Flavonifractor (*Fig. 5.12), an uncultured member of the family Ruminococcaceae (Fig. 5.12) and for *Sutterella* (Fig. 5.13). Significant differences were observed between the Cc extract and FOS for Lachnospiraceae incertae sedis (Fig. 5.12), *Flavonifractor* (Fig. 5.12), uncultured Ruminococcaceae (Fig. 5.12), uncultured Erysipelotrichaceae (Fig 5.12), *Sutterella* (Fig. 5.13), *Comamonas* (Fig. 5.13), *Massilia* (Fig. 5.13), *Enterobacter* (Fig. 5.13), *Klebsiella* (Fig. 5.13), *Pseudomonas* (Fig. 5.13) and an uncultured Pseudomonadaceae genus (Fig. 5.13)

5.5 Discussion.

Seaweeds, in general, are akin to biological metabolite factories. They contain large quantities of polysaccharides and simple sugars as well as vitamins and minerals, proteins and phenolic compounds. Among seaweeds, *C. crispus* has one of the highest percentages of soluble dietary fibre (15-22%) [39]. The predominant polysaccharides found in *C. crispus* are carrageenans, agars and xylans, and it was of interest to see if a polysaccharide extraction of *C. crispus* would exhibit prebiotic activity. The general extraction process for sulphated polysaccharides from red seaweeds is dependent on the purity of the product you wish to obtain. For high purity, the polysaccharide is dissolved in solution, filtered to remove particular matter and then recovered from the solution by precipitation with an organic solvent such as ethanol, a step which also removes large amounts of impurities [19]. Briefly here, a dilute hot-acid (0.1 M HCl) primary extraction was carried out at 70 °C followed by depolymerisation, ethanol precipitation, size exclusion dialysis and a simulated gastric digestion to remove the non-polysaccharide content from the extract. Treatment of the biomass with dilute acid removes the polyvalent cations[40], as well as interrupting the hydrogen bonds between the different cell-wall polysaccharides facilitating an increase in the extraction yield. However care should be taken to prevent the degradation of any acid sensitive polysaccharides [40]. Here, the primary acid extraction of *C. crispus* did not dispel the gelling properties of the Chondrus polysaccharides. When the freeze-dried extract powder was resuspended in minimum deionised water, a strong gel was seen to form. Carrageenans represent one of the main texturizing and gelling agents used by the food industry. They are natural ingredients that have been used for decades in foods and have generally been regarded as safe (GRAS) status [41]. In general, they serve as gelling, stabilising and viscosity-building agents [42]. Any extract for prebiotic studies with such properties could not be added to a fermentation vessel without causing significant technical issues. While it was understood that depolymerisation could greatly degrade the polysaccharides, it was deemed a worthwhile risk in terms of producing an extract that could be successfully added to an anaerobic fermenter for prebiotic investigation. In order to overcome this issue and to decrease the degree of polymerisation, a depolymerisation step was carried out

using hydrogen peroxide [43]. The result of the depolymerisation step was that the gelling effect of the polysaccharide was negated and further processing of the *C. crispus* extract was possible. Ethanol precipitation and high-speed centrifugation were used to segregate the seaweed polysaccharides from the significant quantities of salts and non-polar content present. The next step in processing the extract was to remove simple sugars and any other small components that weren't removed by ethanol precipitation. Monosaccharides, such as glucose, are the preferred energy source for bacteria as the enzymes needed for their degradation and oxidization are widely found. A selective effect on growth would not be seen *ex vivo* with an extract containing high amounts of simple sugars. Size exclusion dialysis is a process used to selectively separate molecules by molecular weight (size exclusion). The partial cleavage of glycosidic bonds is a useful approach for isolation of oligosaccharide fragments in structural analysis of complex polysaccharides [44]. Trifluoroacetic acid was discovered in the early 1900s and is widely used as a solvent, catalyst and a reagent [45]. Treatment of the Cc extract with the TFA resulted in the liberation of large quantities of galactose. In red seaweeds, hydrolysis of carrageenan and agar leads to high amounts of galactose and 3,6-anhydro-galactose in the hydrolysate [4]. From this we conclude that the Cc extract, following extensive post extraction processing, is primarily composed the polysaccharide carrageenan as carrageenan can comprise 50-65% of the dry weight of *C. crispus* [46].

Historically, investigations of the colonic microbiota relied heavily on conventional culturing techniques. These techniques, unfortunately, had an inherent bias, since roughly 90% of the gut microbiota have not yet been cultured [47]. To investigate the prebiotic potential of this extract, an *ex vivo* faecal fermentation approach was taken. Batch fermentation vessels inoculated with faecal slurry are useful for comparative evaluation of putative prebiotics as several can be set up simultaneously and can be used on a small scale for the screening of novel substrates that are only available in small quantities [48]. Here, the MultiFors fermentation system (Infors UK Ltd) was used to mimic the conditions found in the distal colon. When non-digestible carbohydrates are taken in as part of the diet, they pass through the digestive system intact until they reach the colon where the microbiota uses them as substrates for fermentation. To carry out a putative prebiotic study, the environmental conditions of the colon were duplicated as closely as possible. The most

important aspect of the model was to ensure that the environment was completely anaerobic. The bifidobacteria are obligate anaerobes and are not tolerant of oxygen in their environment. To achieve this, N_2 gas was sparged continuously throughout the vessels for the duration of each fermentation run beginning two hours before inoculation with the composite slurry. The pH of each vessel was automatically adjusted so that a constant pH of 6.7 was maintained throughout the fermentation run through the appropriate addition of either NaOH or HCl. SCFAs (acetate, butyrate and propionate) *in vitro* don't accumulate in the colon but instead are absorbed by the colonic mucosa. The contents of each of the vessels were mixed by a magnetic stirrer to keep the mixture of growth media and bacterial cells as homogenous as possible. In a batch fermentation system, SCFAs are not absorbed but remain in the fermentation vessel and the resultant drop in pH could inhibit microbial growth [49]. However, several problems exist with using a batch fermentation system in fermentation studies. Batch fermentations are limited in terms of experimental duration and the amount of substrate added to avoid negative feedback mechanisms. Batch cultures are also highly dependent on the inoculation density as this directly impacts on microbial growth in closed systems. In contrast, continuous culture systems are superior in modelling the dynamic nature of the gastrointestinal tract, allowing the adaptation of various parameters, including dilution rate, retention time, pH and temperature, to meet and maintain optimal growth conditions. Furthermore, substrate replenishment and toxic waste removal are continuous and facilitate studies on the modulation of microbial composition and activity [50]

Short-chain fatty acids are produced as the end products of polysaccharide/oligosaccharide fermentation [48] by microbiota in the colon [51]. They are used by host cells as an energy source as well as acting as regulators of energy intake and energy metabolism [52]. Here, total SCFA production was taken as meaning the accrued production of the short chain fatty acids with between 2 and 6 carbon atoms namely, acetate, propionate, butyrate, iso-butyrate, valerate, isovalerate and hexanoate. There was no significant difference ($P < 0.05$) between the baseline levels of SCFA observed in the different fermentation vessels at 0 h indicating that the microbial load at the beginning of the fermentations was homologous for all fermentation vessels. The main SCFAs of biological importance are acetate, propionate and butyrate, which are produced in the colonic lumen in the approximate

molar ratio of 60:20:20, with production being in the order of acetate> propionate> butyrate [53, 54]. Carbohydrates differ in the extent that they are fermented by microorganisms and in the molar ratio of fatty acids produced, leading to greater or lesser proportions of propionate and butyrate produced, both of which have positive implications in the prevention of colon cancer. Fermentation is also influenced by factors such as the degree of lignification, water solubility, particle size and molecular structure [48].

The approximate molar ratio for the cellulose control after was 58:19:24 which broadly conformed to other reported studies. The ratio for the Cc extract fermentation was measured as 58:24:18, indicating a significantly increased ($p = 0.020$) proportion of propionate production in comparison with the control fermentation. A significant decrease in the ratio of butyrate production was also observed at both 5 h and 10 h respectively. Supplementation with FOS had no apparent effect on the ratio of acetate: propionate: butyrate production. This indicates that the addition of the Cc extract had a propiogenic effect on the *ex vivo* microbial populations. The results obtained from the GC-FID analysis indicate that the depolymerized *C. crispus* extract was poorly metabolised in general by the microbial populations present in the fermentation vessels. The polysaccharides present in the extract, mainly carrageenan, only caused a moderate increase in the production of short-chains. This is in agreement with a study by Ferguson and Jones [54] where the fermentability of 31 carbohydrates (measuring total SCFA production) were graded and carrageenan was placed 30 out of 31. The amounts and proportions of SCFAs were similar to the controls used in the study indicating that carrageenan had undergone little or no fermentation. The generation of SCFAs is one the most important metabolic functions of the microbiota that benefits the host organism. Their creation is an important indicator of a putative prebiotic compound. In contrast with the Cc extract, the FOS was readily fermented by the *ex vivo* bacterial populations resulting in a sharp increase in the total production of SCFAs as well as the individual increases in acetate, propionate and butyrate, the three most biologically relevant SCFAs. The depolymerisation step in the preparation of the Cc extract may have degraded the polysaccharides to such an extent that they could be used as a precursor for SCFA production.

The human microbiota is a highly complex bacterial community dominated by hundreds of species of obligate anaerobes, among which are the bifidobacteria. Many studies have focused on measuring their abundance as an indication of prebiotic effect [55] as their selective stimulation can have several benefits such as the stabilization of the gut mucosal barrier and the prevention of diarrhoea. Bifidobacteria produces both lactic and acetic acids that results in a lowering of luminal pH and the inhibition of pathogens[56]. Like most intestinal bacterial, bifidobacteria are saccharolytic organisms, obtaining their energy through the fermentation of carbohydrates. They play an important role in carbohydrate fermentation in the colon as they possess a wide array of carbohydrate modifying enzymes. For example, 5% of the whole genome of *Bifidobacterium longum* corresponds to carbohydrate-modifying enzymes. Bifidobacteria use a variety of glycolyl hydrolases to degrade a variety of plant polysaccharides or host-derived glycoproteins and glycol-conjugates that release a variety of mono- and oligosaccharides that are then catabolised [57, 58]. The selective stimulation of growth of beneficial members of the gut microflora is a key feature of the prebiotic definition coupled with the idea of augmenting their metabolic activity. As measured by bacterial plate counts, fermentation of the *C. crispus* polysaccharides had no impact on the growth of Bifidobacteria and a detrimental effect on the growth of Lactobacilli. In comparison, the FOS control (a known prebiotic) has a notable, if not significant, impact on numbers of Bifidobacteria. FOS is composed of a mixture of fucose moieties that are linked by β-(2-1) glycosidic bonds with a terminal glucose unit. Bifidobacteria possess a β- fructofuranosidase enzyme that hydrolyses fructose moieties from the terminal β-2,1 position and is involved in the hydrolysis of fructans and sucrose [57]. By this mechanism, bifidobacteria can utilize FOS and other inulin type compounds for growth.

The classical microbiological approach to the cultivation and study of microorganisms began in the 17th century when a Dutch linen merchant constructed the first apparatus capable of viewing the microbial world that is all around us. Since then advances in technology has allowed scientists to move past culture-dependent studies and towards non-culture based technologies such as 16S rRNA sequencing which has provided extensive information on the microbial composition of many different ecosystems. The 16s rRNA gene is the most common target for sequencing studies as it is present in all prokaryotes and contains hypervariable regions that allow

for distinction to be made between the different taxa [59]. Here the V4 variable region of the 16s rRNA gene was targeted for high throughput DNA sequencing using a 454 Genome Sequencer FLX platform. The prebiotic concept is centered on the notion that the ingestion of non-digestible, fermentable food ingredients can stimulate the growth and/or activity of a small number of beneficial members of the gut microbiota. While not named specifically, this is taken to mean stimulation of *Bifidobacterium* and *Lactobacillus* and an increasing number of other beneficial bacteria such as *Roseburia*.

As expected, large increases in the *ex vivo* populations of Bifidobacteria were observed during the FOS fermentation. However, there were no likewise increases of Bifidobacteria brought about by the Cc extract. Further, neither the FOS nor the Cc extract had any significant effect on the relative abundance of *Lactobacillus*. These observations positively correlate with the data recovered from the major probiotic genera plate counts. It would appear the Cc extract did not stimulate the growth of either of the two main probiotic genera. At the genus level, all three fermentation conditions (cellulose control, FOS and Cc extract) brought about notable decreases in *Faecalibacterium*, another species often mentioned as being probiotic in nature. A substantial reduction in the relative abundance of *Klebsiella* was observed in the Cc supplemented vessels. *Klebsiella* is an opportunistic pathogen with the majority of infections being associated with hospitalization. Those most at risk are immunocompromised individuals who have been hospitalized and suffer from a severe underlying disease such as diabetes and chronic pulmonary obstruction. Nosocomial acquired infections are mainly caused by *Klebsiella pneumonia* [60]

Rarefaction curves were calculated at 97% similarity and were saturated for all samples, indicating that extra sampling would only yield a limited increase in species richness. A scatter plot analysis of alpha diversity indicated that neither the *C. crispus* extract nor the FOS had a substantial influence on species diversity. Beta diversity was estimated using principal coordinate analysis (PCA) plots generated using an unweighted Unifrac distance matrix. Through PCA, the samples were shown to cluster into relatively distinct groupings based on the fermentation run and not treatment type indicating that the greatest factor affecting diversity was the initial composition the faecal slurry inoculum. A study carried out by Liu et al., [46] looked at the prebiotic effect of *C. crispus* supplemented diets in rats. The study found that the

supplementation with *C. crispus* (2.5%) resulted in significant increases in the population of *Bifidobacterium breve* and a reduction of the abundance of the pathogenic species *Clostridium septicum* and *Streptococcus pneumonia*. Also, higher concentration of short-chain fatty acids was found in faecal samples of the *C. crispus* group. The *C. crispus* used in this study was not a refined extract as presented here. No processing was carried out to isolate the polysaccharide content and to remove non-carbohydrate matter. The study also found that the observed effect was dose dependent with a 2.5% concentration being optimal. This is a higher concentration than that used in this investigation and suggests that a slightly increased percentage of extract could yield an increase in observed prebiotic activity.

5.6 Conclusion

The three groupings of seaweeds, red seaweeds (phylum Rhodophyta), brown seaweeds (phylum Ochrophyta, class Phaeophyceae) and green seaweeds (phylum Chlorophyta) contain large quantities of polysaccharides that resist digestion in the mammalian digestive tract and thus are potential novel sources of prebiotic compounds. Seaweeds of the phylum Rhodophyta contain large quantities of polysaccharides such as carrageenan, agarans and xylans which differ from the main polysaccharides found in the brown seaweed (laminarin, fucoidan, alginate) and the green seaweeds (ulvan). The concept of prebiotics is an intriguing one. The delivery of non-digestible components to the gut, after surviving gastric transit, and then being utilised by certain beneficial members of the microbiota, namely *Bifidobacterium* and *Lactobacillus*. However, not all putative prebiotics are made equally and they vary considerably in their fermentability potential. The conclusion of this study is that a polysaccharide-rich extract from *C. crispus*, prepared as outlined above, does not exhibit prebiotic activity when used as the sole source of carbon in an *ex vivo* fermentation model. The results obtained from high throughput DNA sequencing and traditional enumeration techniques clearly indicate that the fermentation of *C. crispus* extract failed to selectively stimulate the main probiotic genera of *Bifidobacterium* and *Lactobacillus* and had a minimum effect on the overall microbial population. The production of SCFAs, especially the biologically important acids acetate, butyrate and propionate, are an excellent indication of stimulation of microbial activity. Increases in propionate concentration, while statistically significant ($p<0.05$) at certain time points, fell short of the levels that would be expected from a putative prebiotic. This study concludes that depolymerised *C. crispus* polysaccharides were poorly fermented by the *ex vivo* microbiota and did not have a significant impact on the microbial populations present. It is reasonable to assume that depolymerisation with hydrogen peroxide resulted in degradation of the red seaweed polysaccharide. While the *C. crispus* extract, in this form, does not exhibit prebiotic activity it would be of great interest to revisit this seaweed using different approaches to overcome the gelling properties of carrageenans such as an enzymatic extraction approach rather than hot acid extraction and depolymerisation.

5.7 References

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Figure 5.1. Compositional analysis of the Cc extract. The Cc extract was treated the TFA and the concentrations of glucose and galactose were determined.

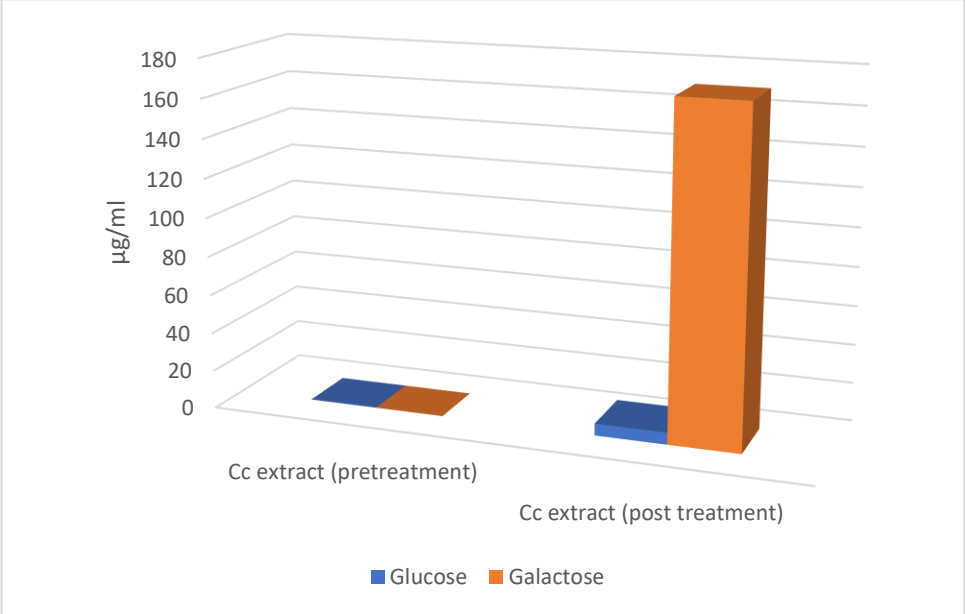
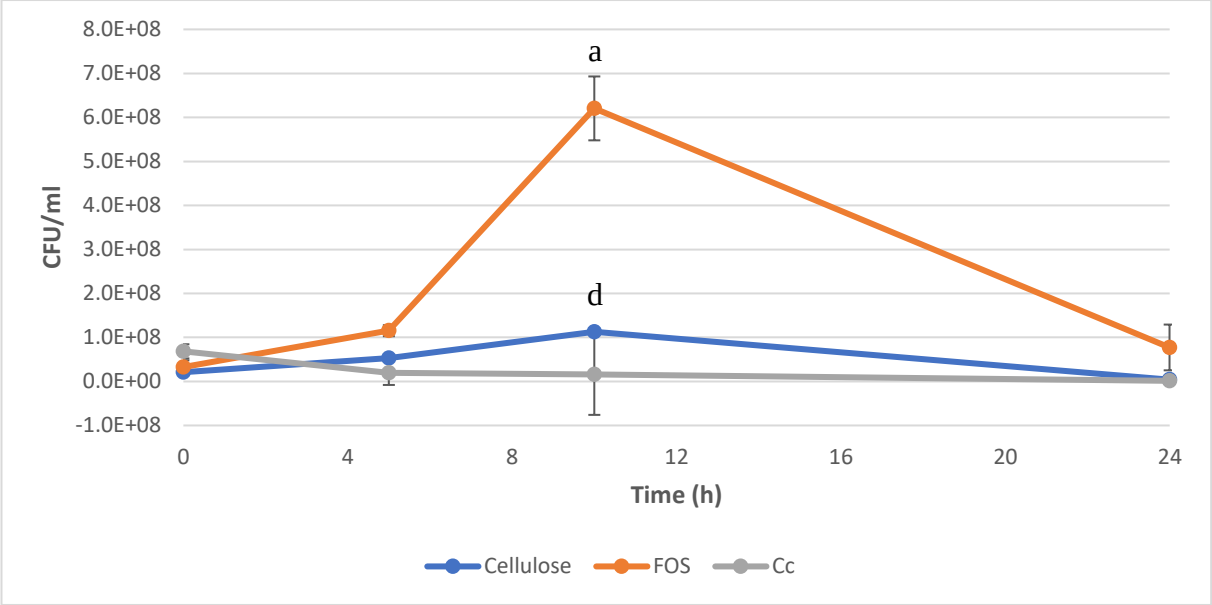


Figure 5.2. Plate count data for (a) culturable *Bifidobacterium* and (b) culturable *Lactobacillus* populations at 0 h, 5 h, 10 h and 24 h. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

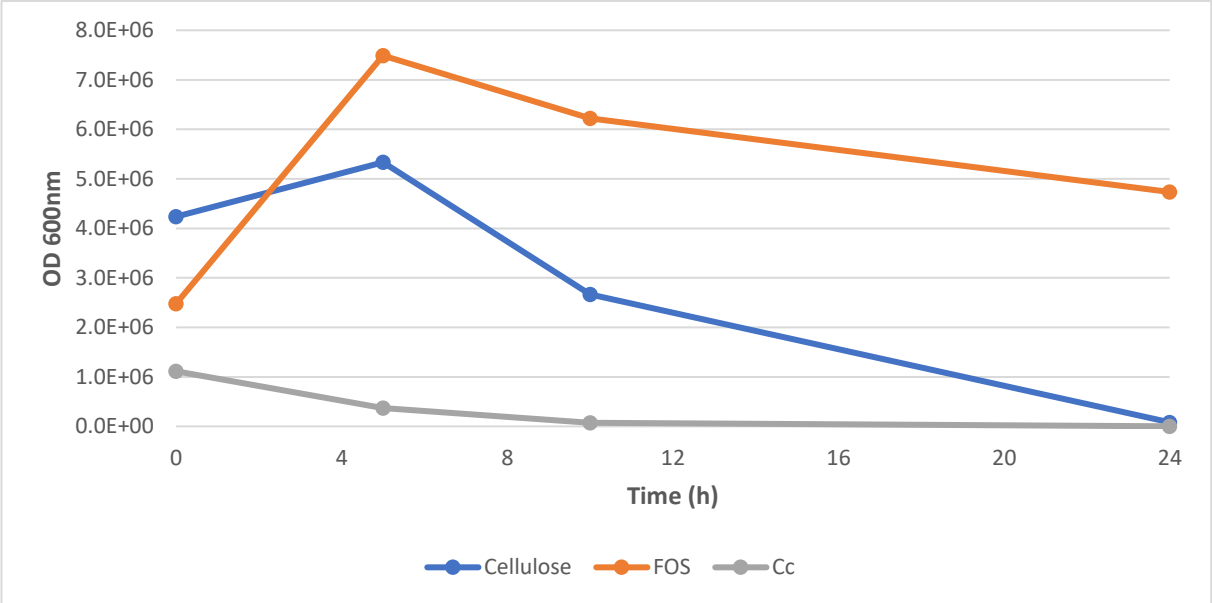
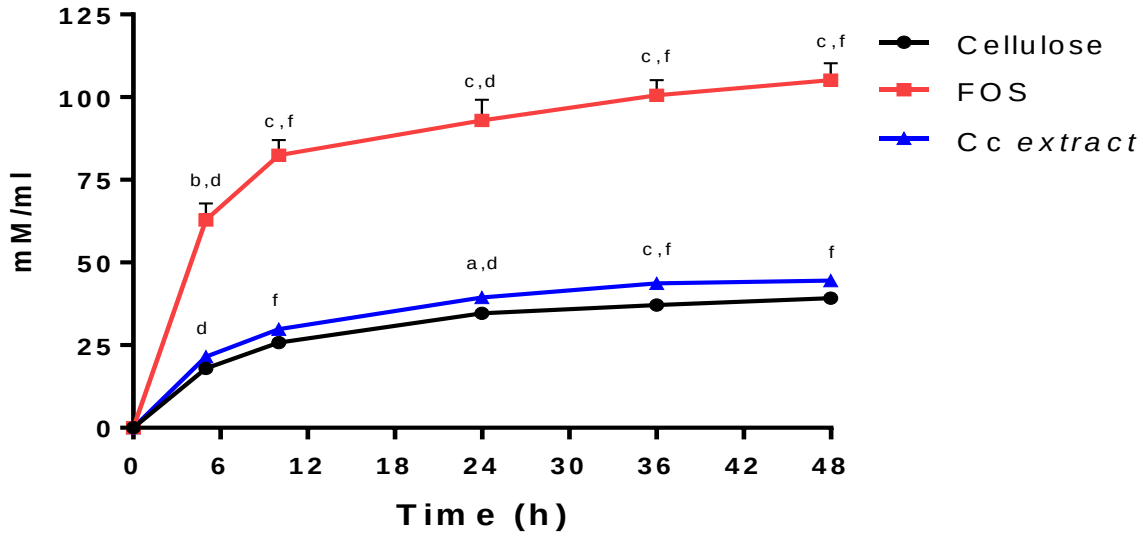


Figure 5.3 Total SCFA production. The effect of the *C. crispus* extract and FOS on (a) total SCFA concentration and (b) SCFA production. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

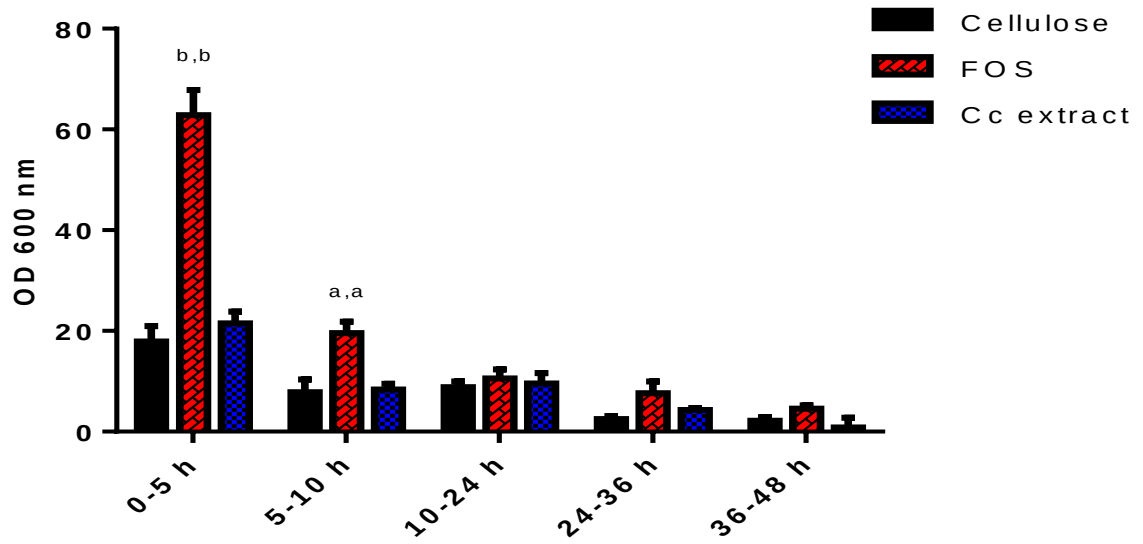
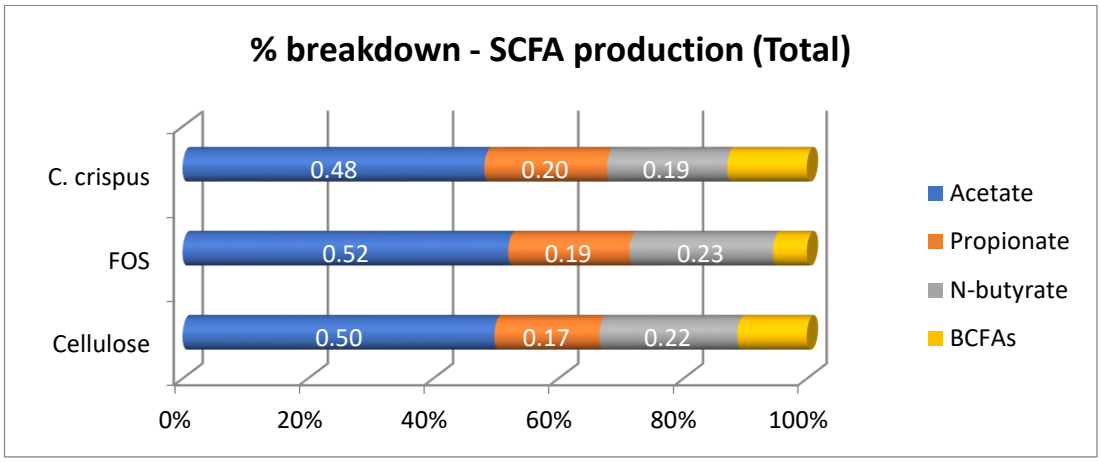


Table 5.1 Effect of Cc extract fermentation on the molar ratio of SCFA production.

	A:P:B	**A:P:B:BC**
Total SCFA production		
Cellulose	56:19:25	50:17:22:11
FOS	55:21:25	52:19:23:6
C. crispus extract	55:22:22	48:20:19:13
Production 0 – 24 h		
Cellulose	57:19:24	52:17:21:10
FOS	56:21:23	55:20:22:4
C. crispus extract	58:24:18	52:23:14:8
Production 24 - 48 h		
Cellulose	39:20:41	28:15:29:28
FOS	42:15:43	33:12:33:22
C. crispus extract	29:13:57	23:10:38:29

Figure 5.4 Breakdown of SCFA production by time. Percentage breakdown of (a) total SCFA, and (b) SCFA production 0 – 24 h.

(a)



(b)

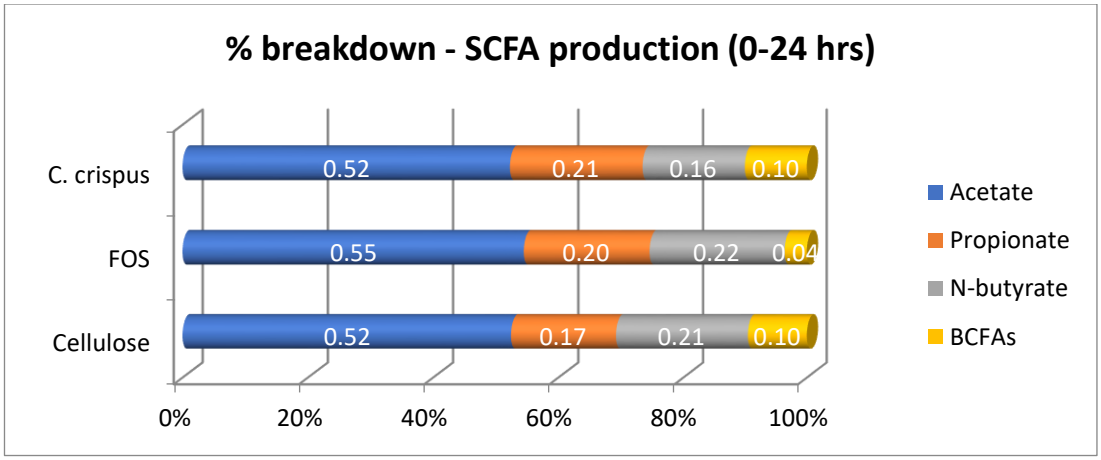
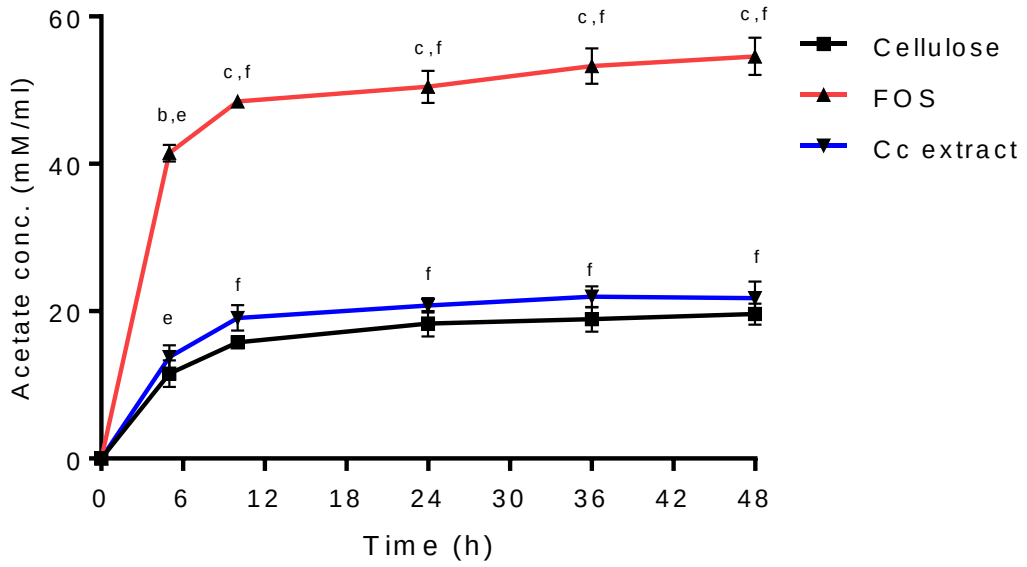


Figure 5.5 Acetate production. The effect of the *C. crispus* extract and FOS on (a) acetate concentration and (b) acetate production. Baseline values obtained at 0 h were subtracted from each time point Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired T-test).

(a)



(b)

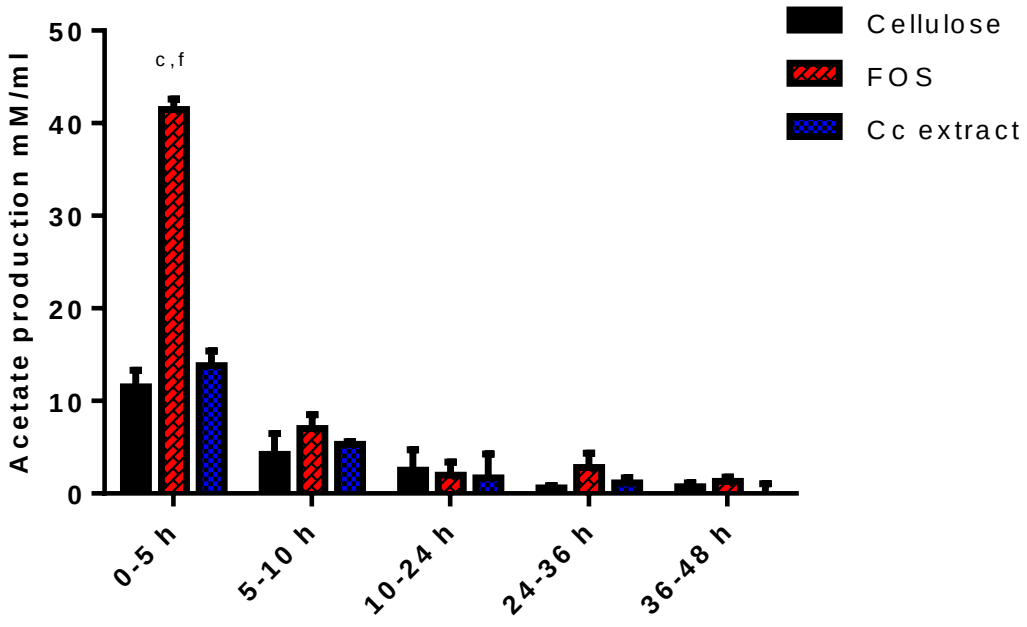
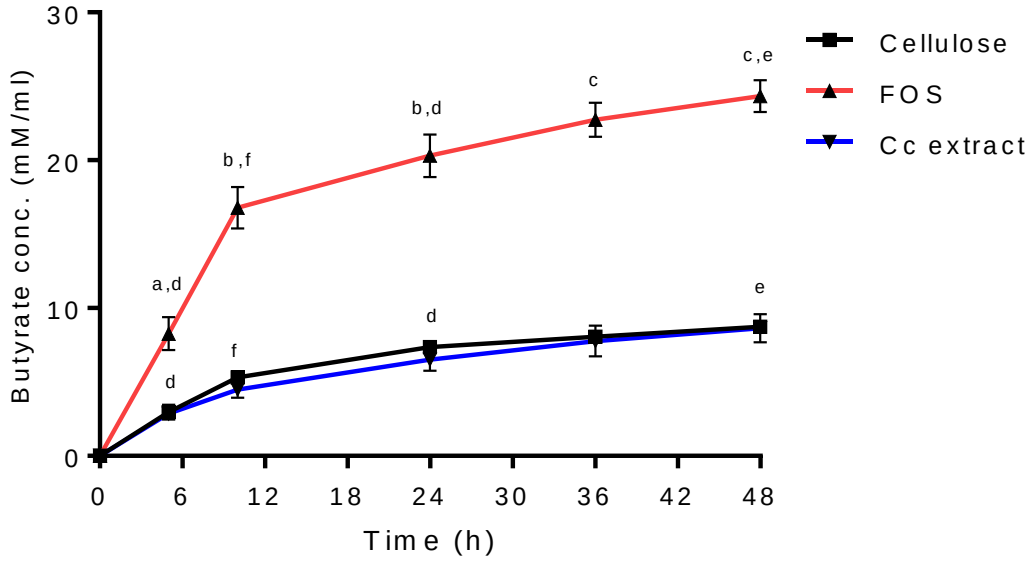


Figure 5.6 Butyrate production. Effect of the *C. crispus* extract and FOS on (a) butyrate concentration and (b) butyrate production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.0005 relative to FOS, un-paired t-test).

(a)



(b)

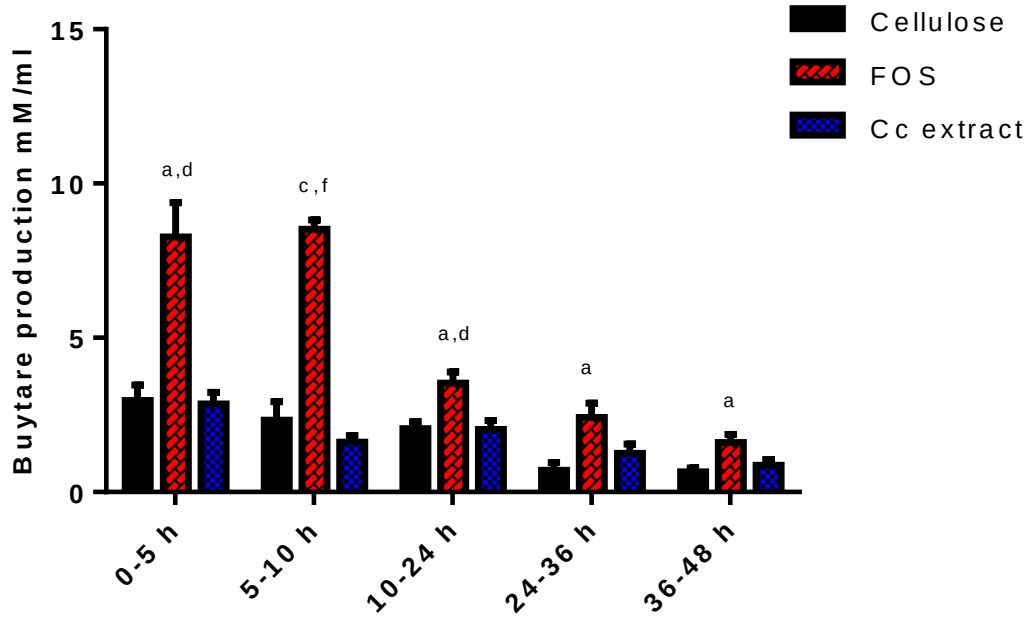
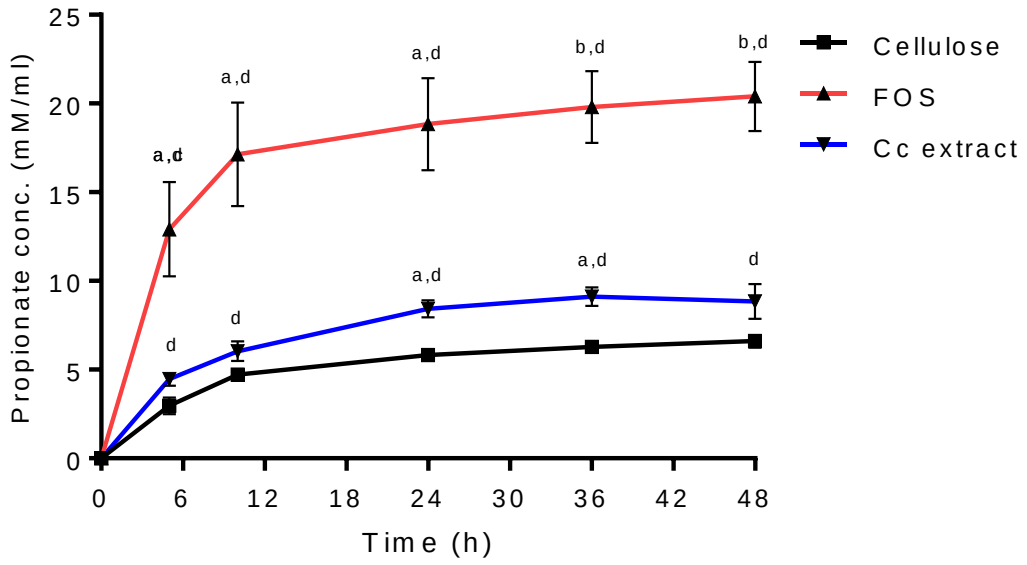


Figure 5.7 Propionate production. The effect of the *C. crispus* extract and FOS on (a) propionate production and (b) propionate production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test).

(a)



(b)

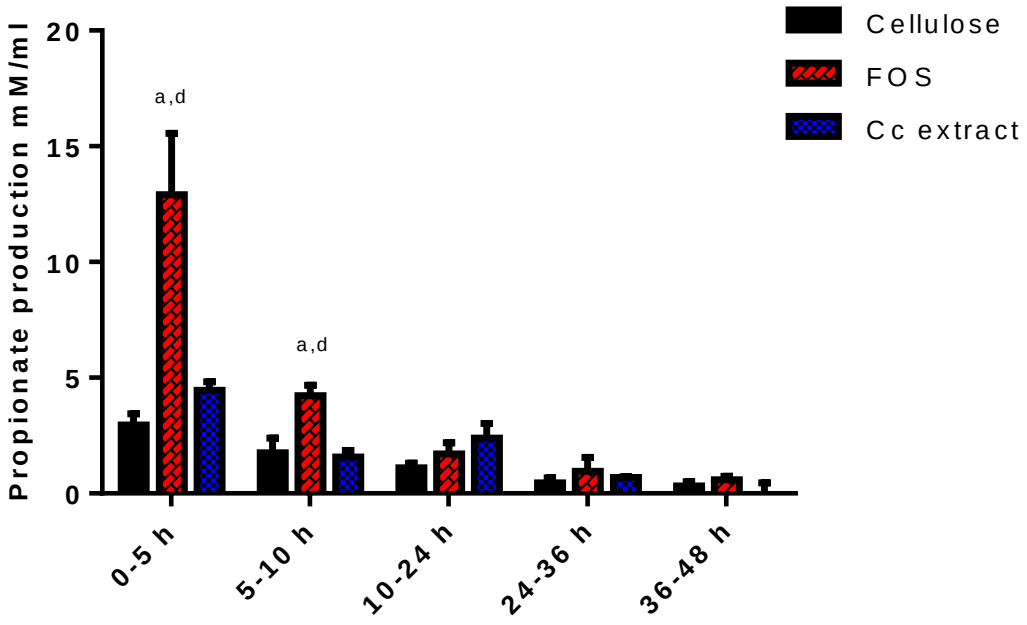
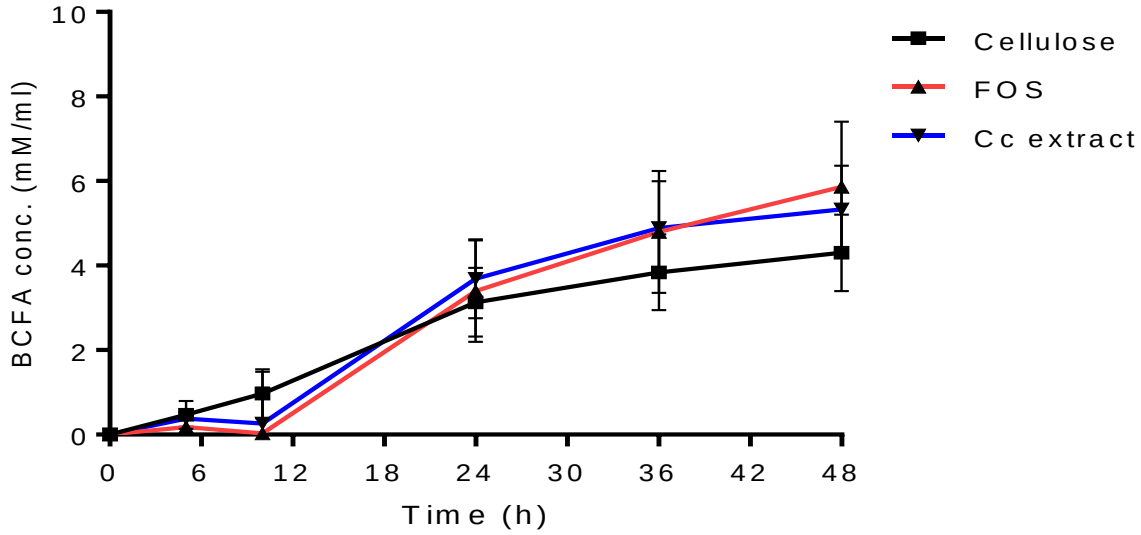


Figure 5.8 Branched-chain fatty acid production. The effect of the *C. crispus* extract and FOS on (a) BCFA concentration (b) BCFA production. Baseline values obtained at 0 h were subtracted from each time point. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to cellulose, d = P<0.05, e = P<0.005, f = P<0.005 relative to FOS, un-paired t-test).

(a)



(b)

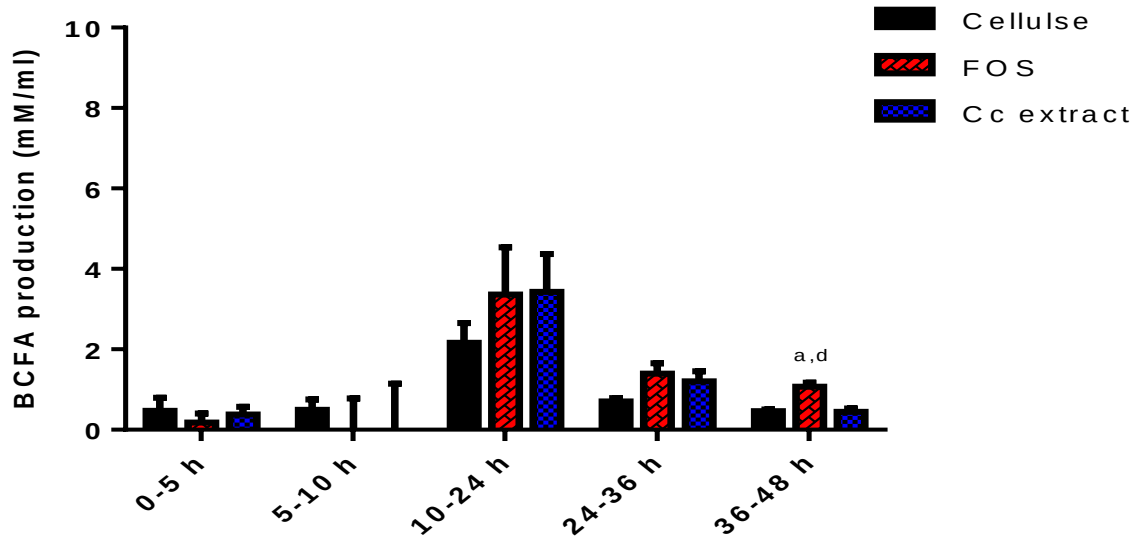
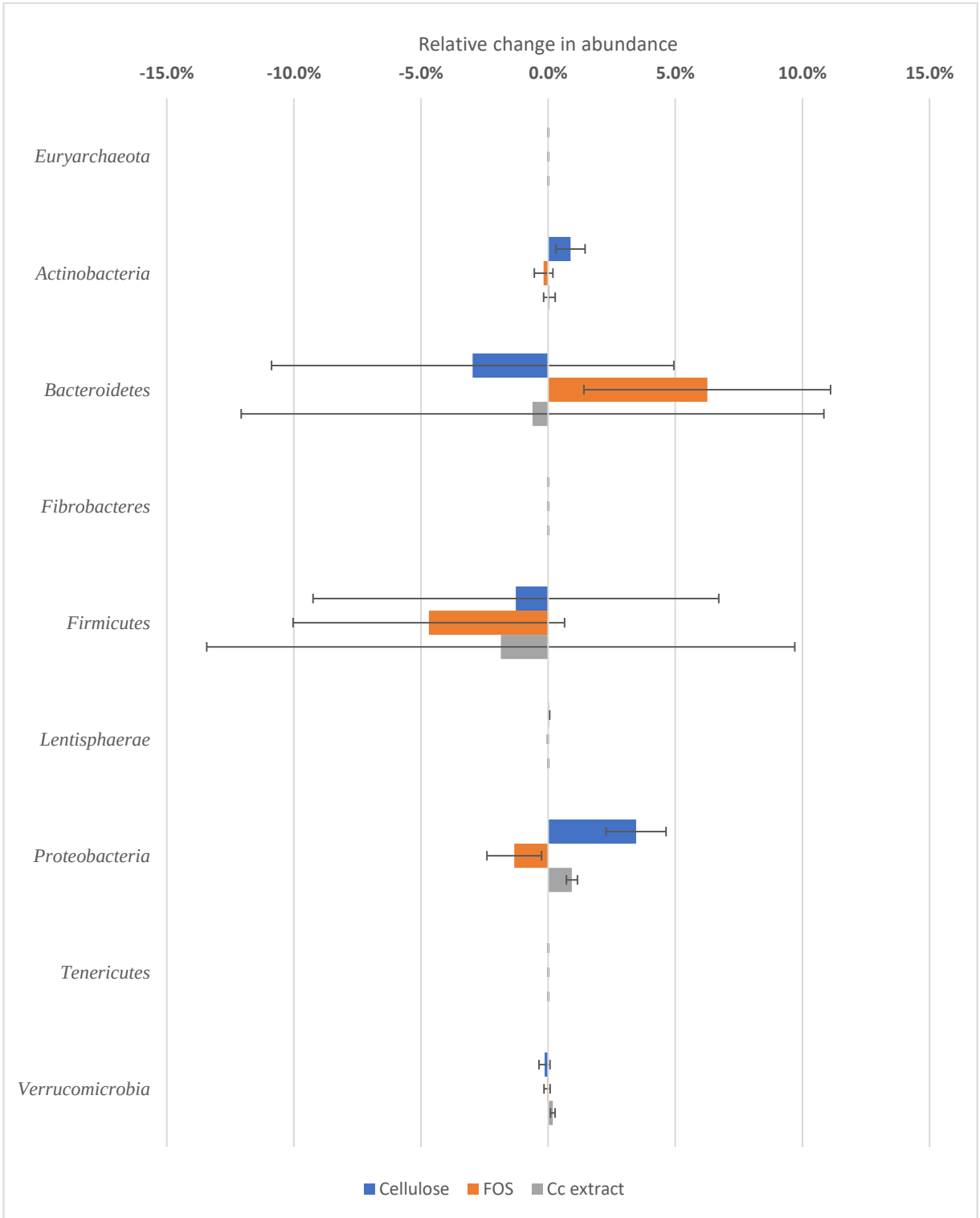


Figure 5.9 (a) Increase/decrease in relative abundance at the phylum level. (b) Percentage change in relative abundance at the phylum level. Data represent the mean (±SE).

(a)



(b)

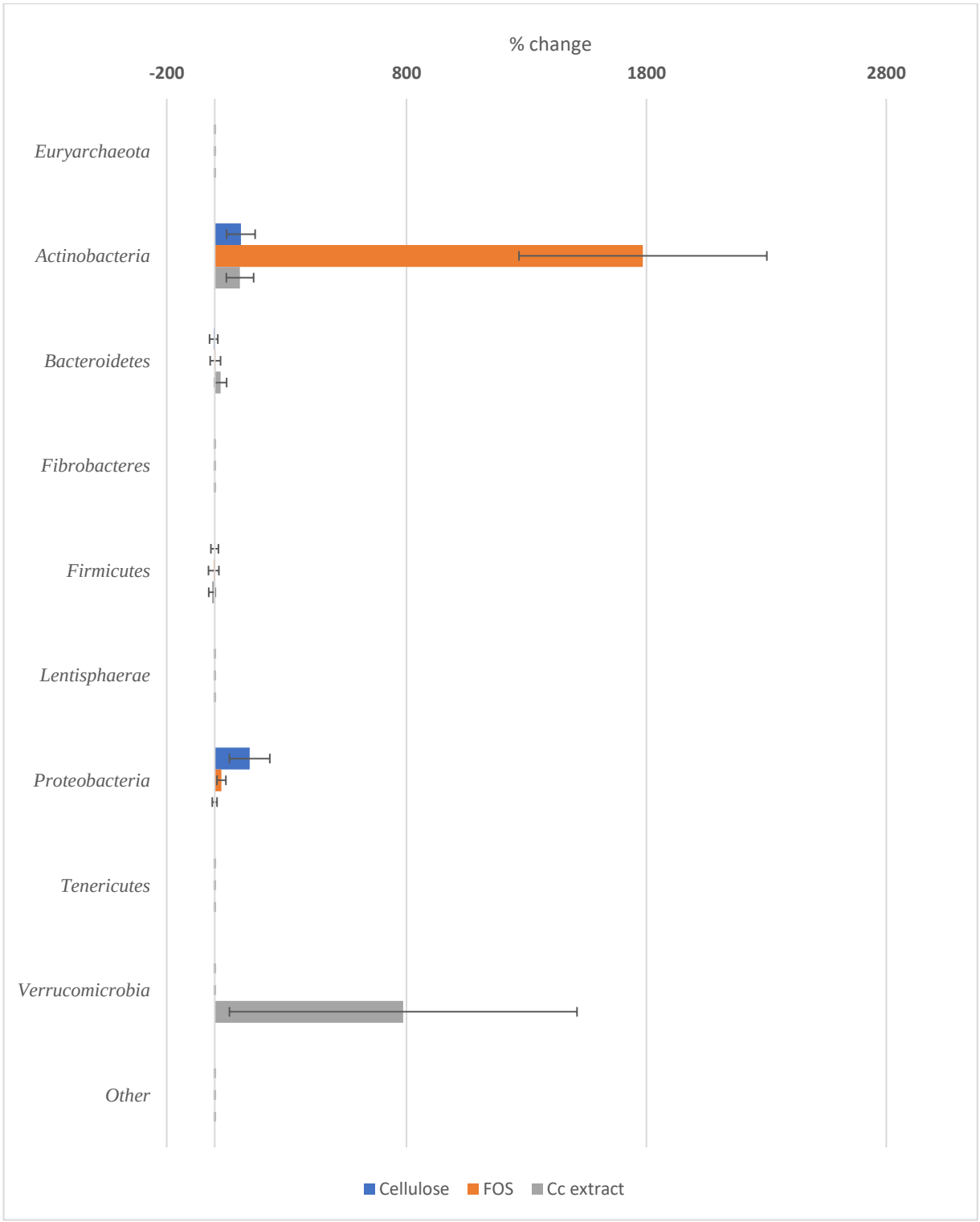
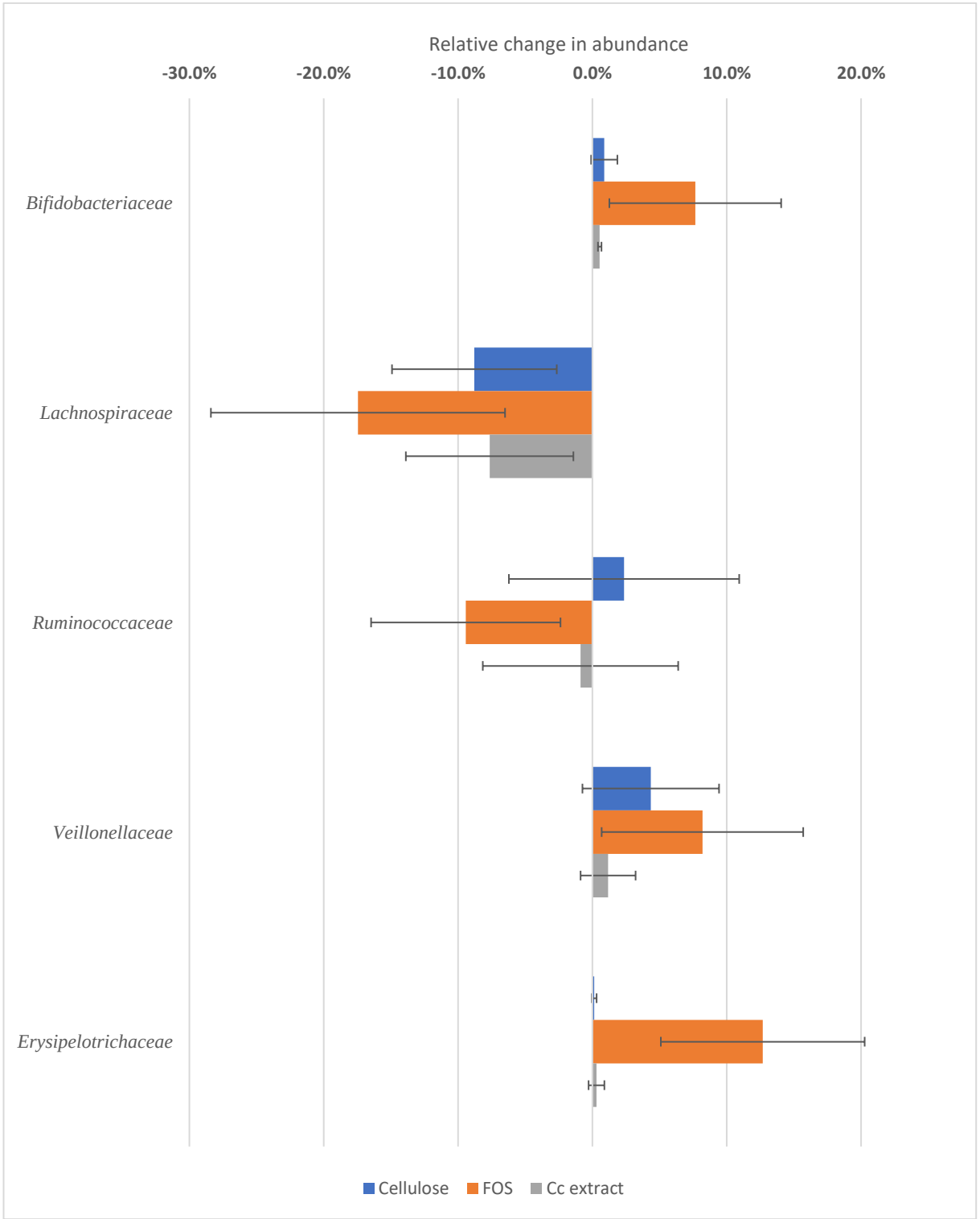
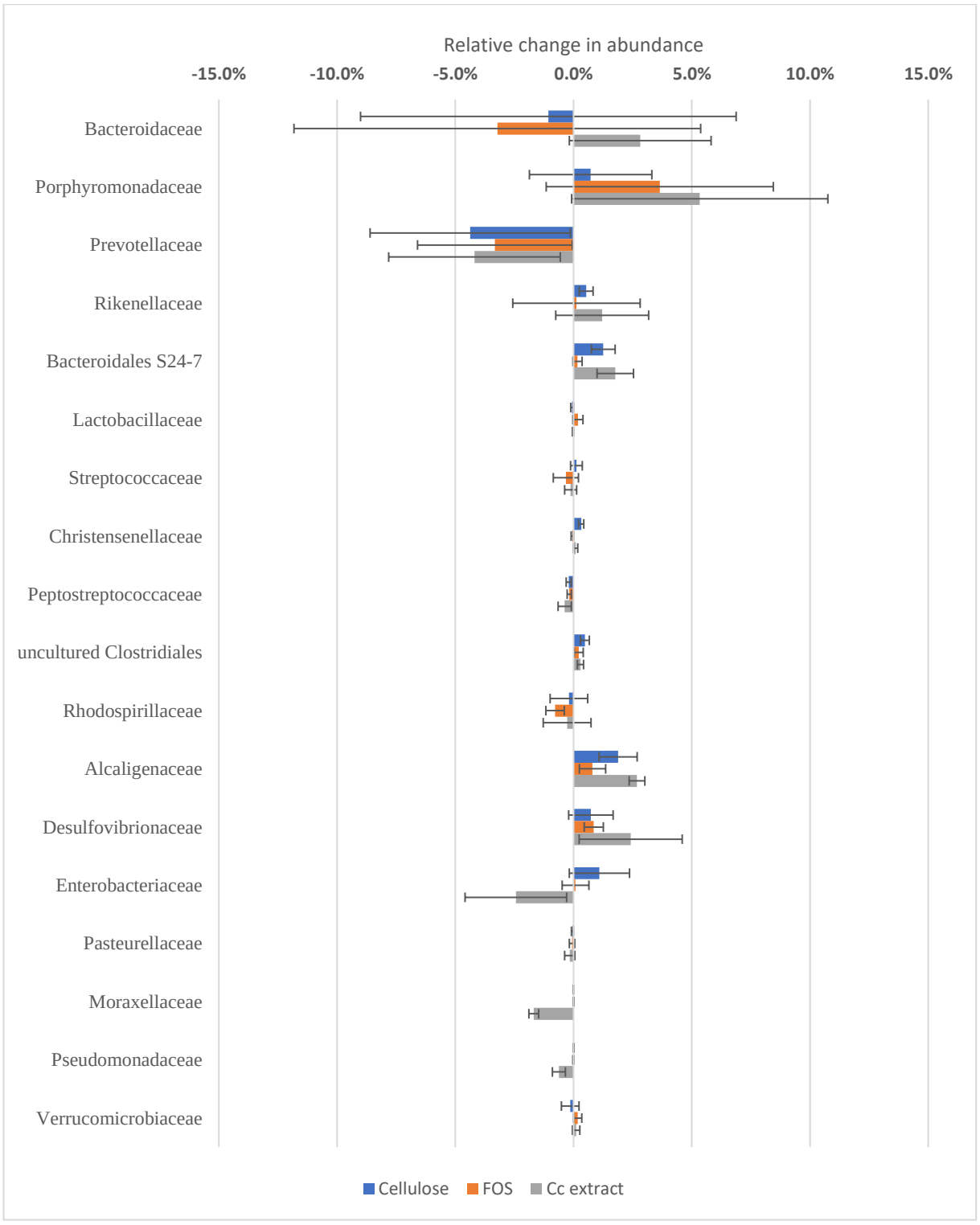

% change
-200
800
1800
2800
Euryarchaeota
Actinobacteria
Bacteroidetes
Fibrobacteres
Firmicutes
Lentisphaerae
Proteobacteria
Tenericutes
Verrucomicrobia
Other
Cellulose
FOS
Cc extract

Figure 5.10 (a) Notable increases/decreases in relative abundance at the family level. (b) Percentage change in the relative abundance at the family level. Values represent the mean (±SE).

(a)




Relative change in abundance
-15.0%
-10.0%
-5.0%
0.0%
5.0%
10.0%
15.0%
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Bacteroidales S24-7
Lactobacillaceae
Streptococcaceae
Christensenellaceae
Peptostreptococcaceae
uncultured Clostridiales
Rhodospirillaceae
Alcaligenaceae
Desulfovibrionaceae
Enterobacteriaceae
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Verrucomicrobiaceae
Cellulose
FOS
Cc extract

(b)

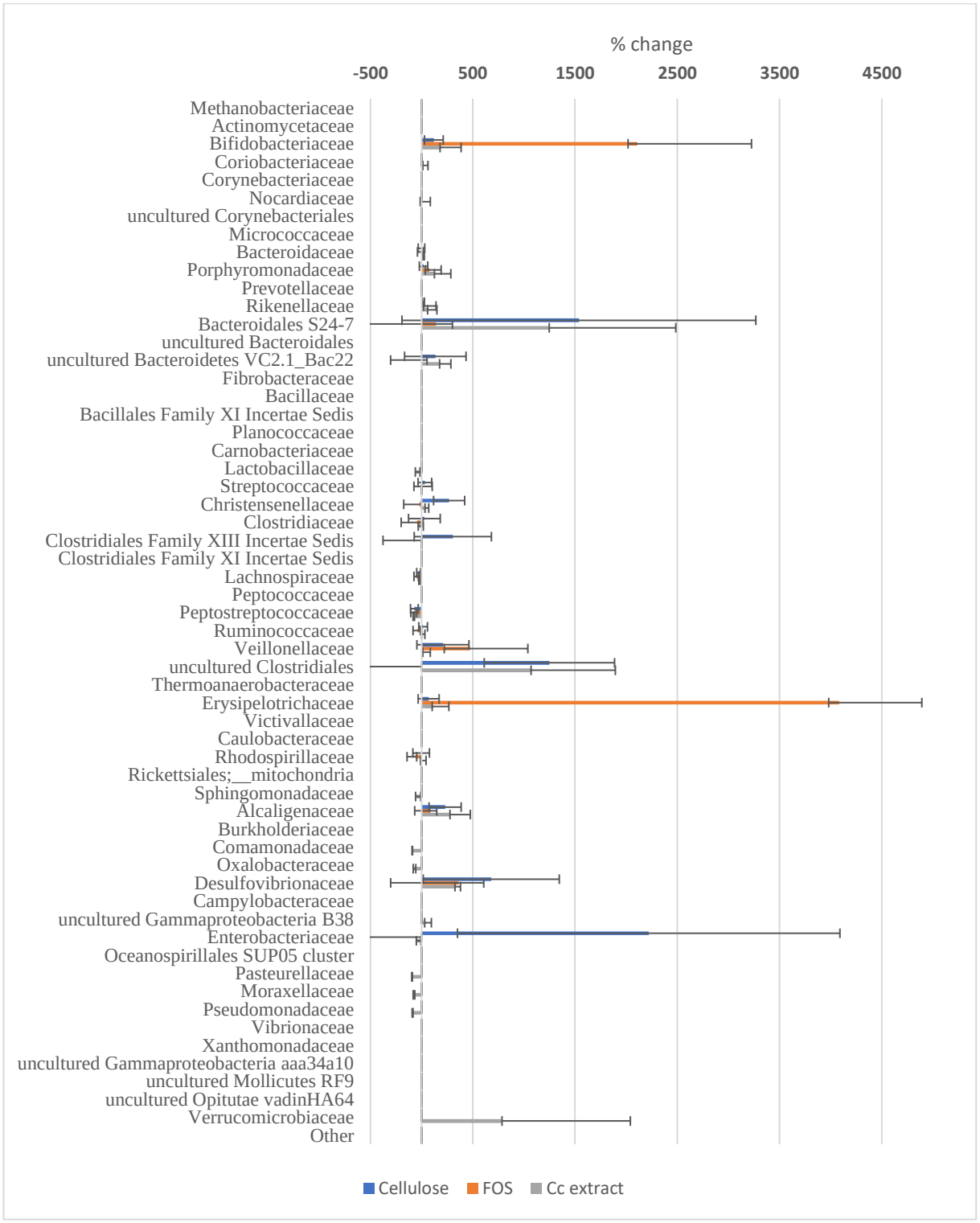
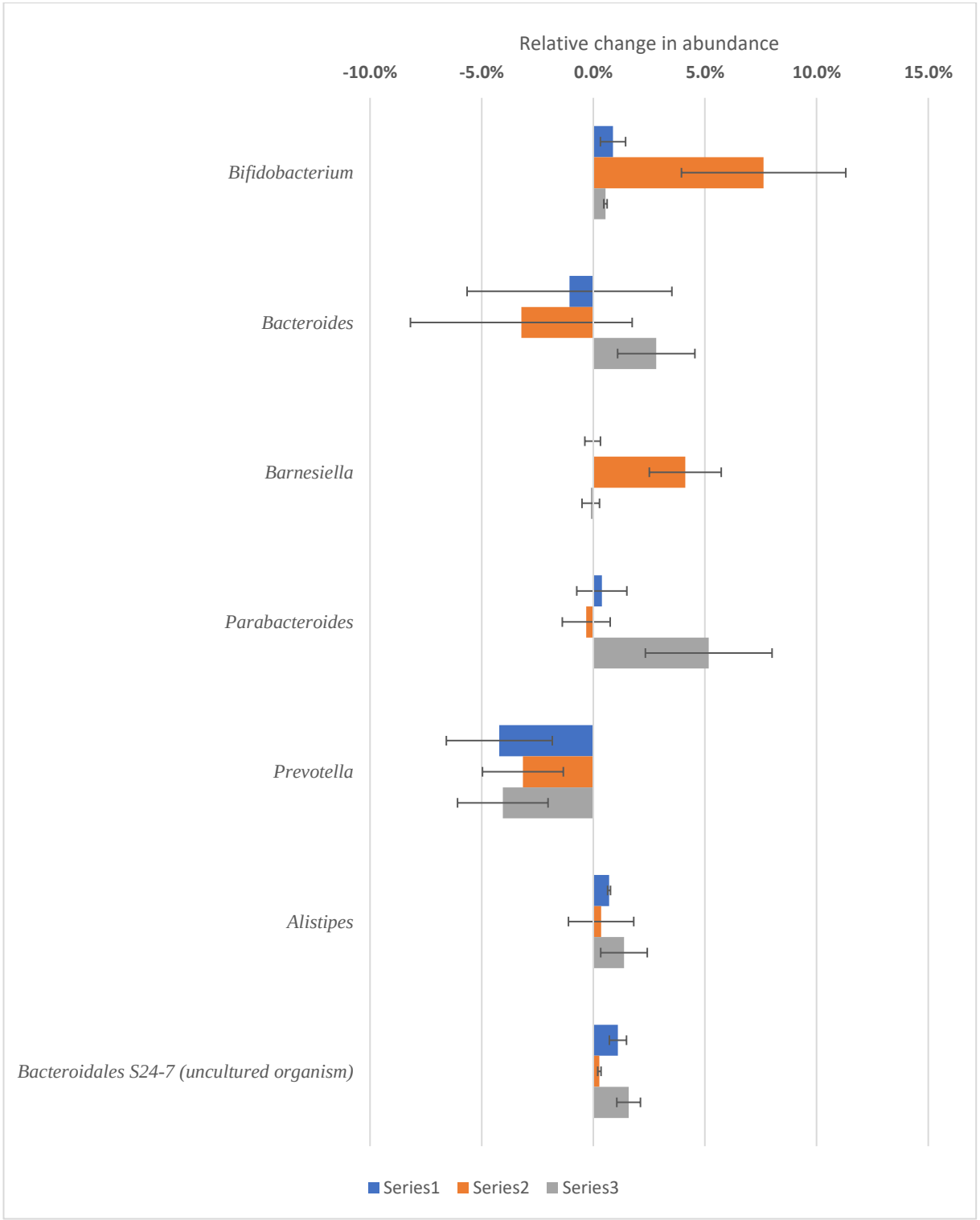
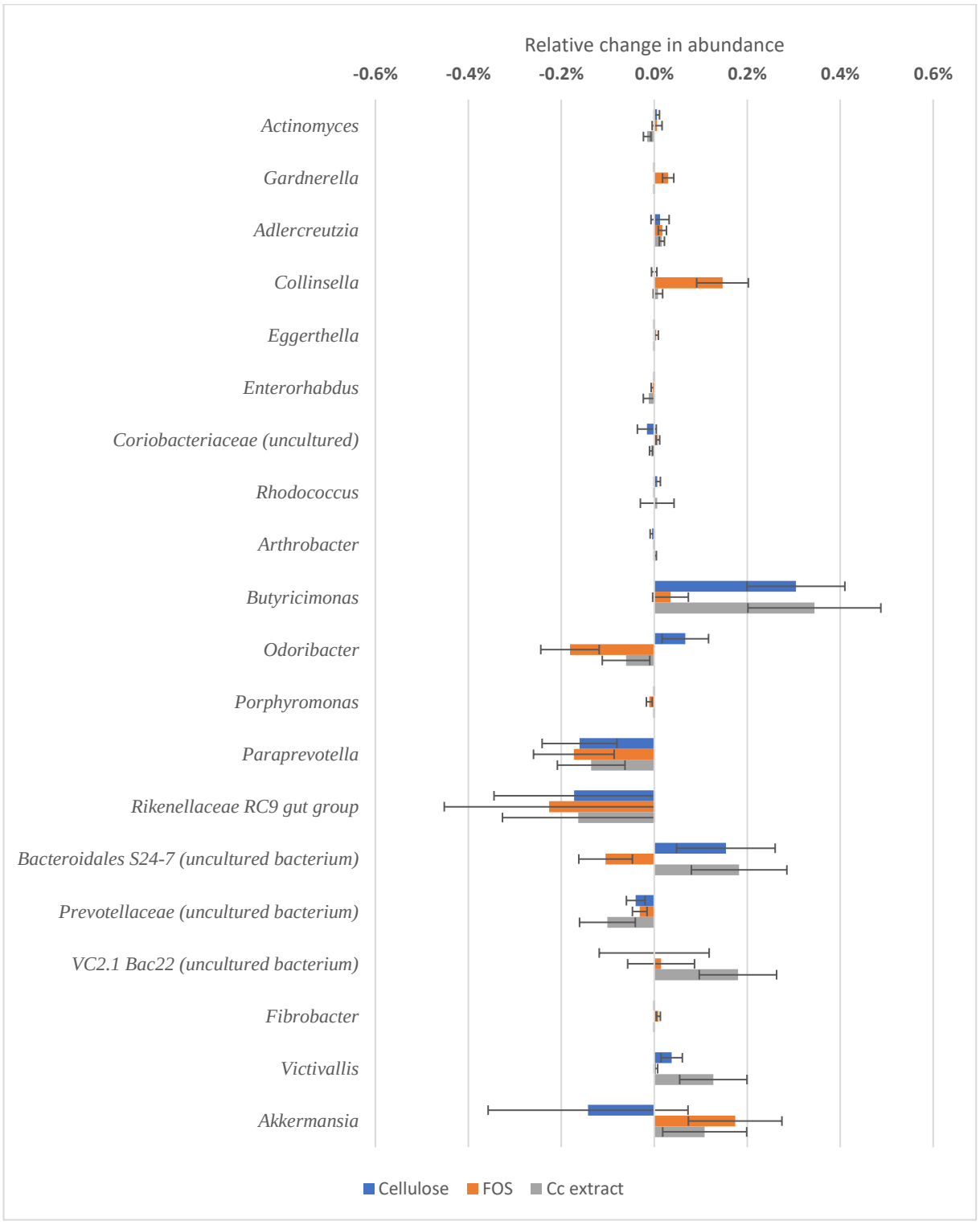

% change
-500
500
1500
2500
3500
4500
Methanobacteriaceae
Actinomycetaceae
Bifidobacteriaceae
Coriobacteriaceae
Corynebacteriaceae
Nocardiaceae
uncultured Corynebacteriales
Micrococcaceae
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Bacteroidales S24-7
uncultured Bacteroidales
uncultured Bacteroidetes VC2.1_Bac22
Fibrobacteraceae
Bacillaceae
Bacillales Family XI Incertae Sedis
Planococcaceae
Carnobacteriaceae
Lactobacillaceae
Streptococcaceae
Christensenellaceae
Clostridiaceae
Clostridiales Family XIII Incertae Sedis
Clostridiales Family XI Incertae Sedis
Lachnospiraceae
Peptococcaceae
Peptostreptococcaceae
Ruminococcaceae
Veillonellaceae
uncultured Clostridiales
Thermoanaerobacteraceae
Erysipelotrichaceae
Victivallaceae
Caulobacteraceae
Rhodospirillaceae
Rickettsiales;__mitochondria
Sphingomonadaceae
Alcaligenaceae
Burkholderiaceae
Comamonadaceae
Oxalobacteraceae
Desulfovibrionaceae
Campylobacteraceae
uncultured Gammaproteobacteria B38
Enterobacteriaceae
Oceanospirillales SUP05 cluster
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Vibrionaceae
Xanthomonadaceae
uncultured Gammaproteobacteria aaa34a10
uncultured Mollicutes RF9
uncultured Opitutae vadinHA64
Verrucomicrobiaceae
Other
Cellulose
FOS
Cc extract

Figure 5.11 (a) a) Increase/decrease in relative abundance of genera in the phyla Actinobacteria, Fibrobacteres, Lentisphaerae, Proteobacteria, Tenericutes, and Verrocomicrobia. (b) Percentage change in the relative abundance of genera in the phyla Actinobacteria, Fibrobacteres, Lentisphaerae, Proteobacteria, Tenericutes, and Verrocomicrobia. Data represent the mean (±SE).

(a)




Relative change in abundance
-0.6%
-0.4%
-0.2%
0.0%
0.2%
0.4%
0.6%
Actinomyces
Gardnerella
Adlercreutzia
Collinsella
Eggerthella
Enterorhabdus
Coriobacteriaceae (uncultured)
Rhodococcus
Arthrobacter
Butyricimonas
Odoribacter
Porphyromonas
Paraprevotella
Rikenellaceae RC9 gut group
Bacteroidales S24-7 (uncultured bacterium)
Prevotellaceae (uncultured bacterium)
VC2.1 Bac22 (uncultured bacterium)
Fibrobacter
Victivallis
Akkermansia
Cellulose
FOS
Cc extract

(b)

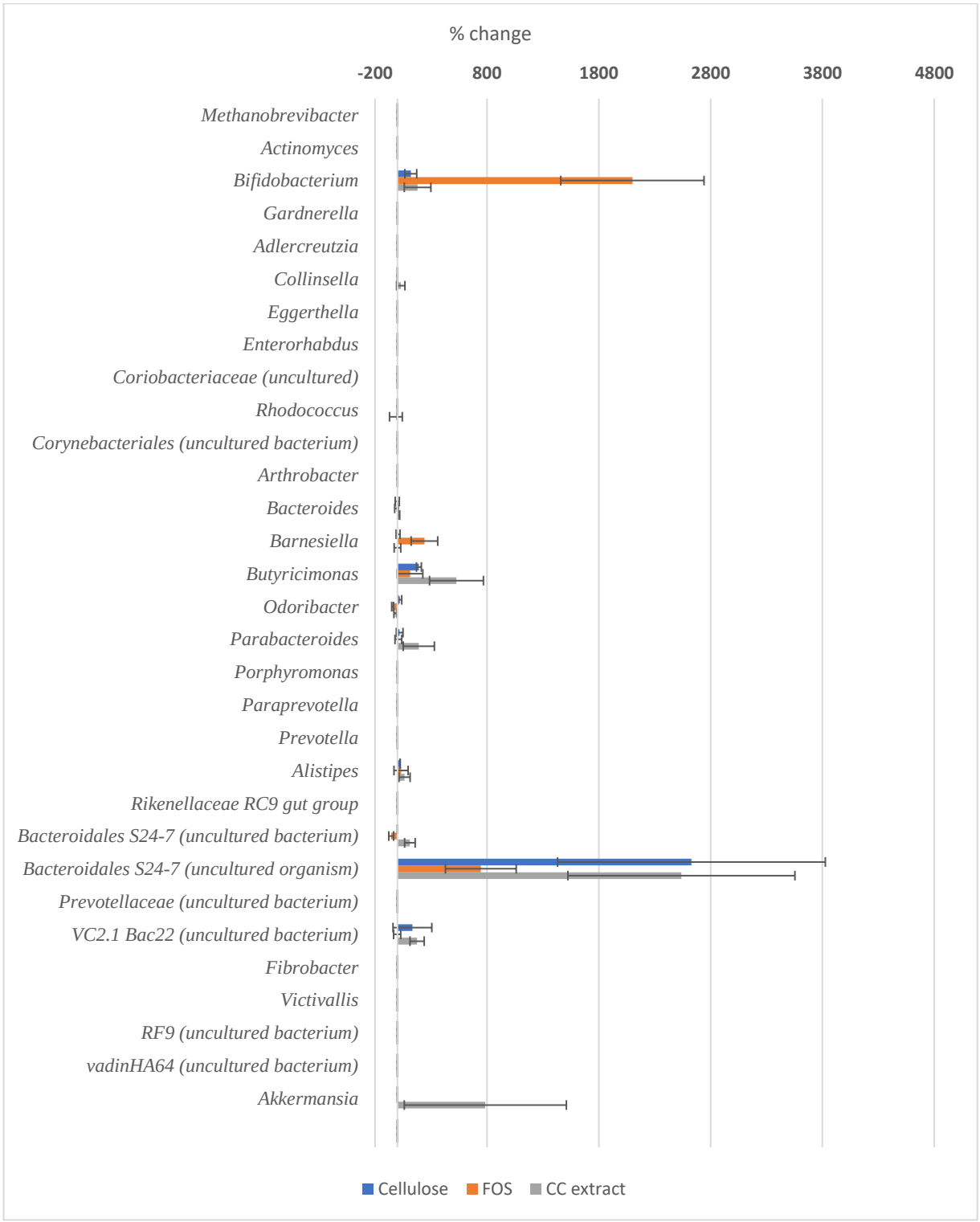
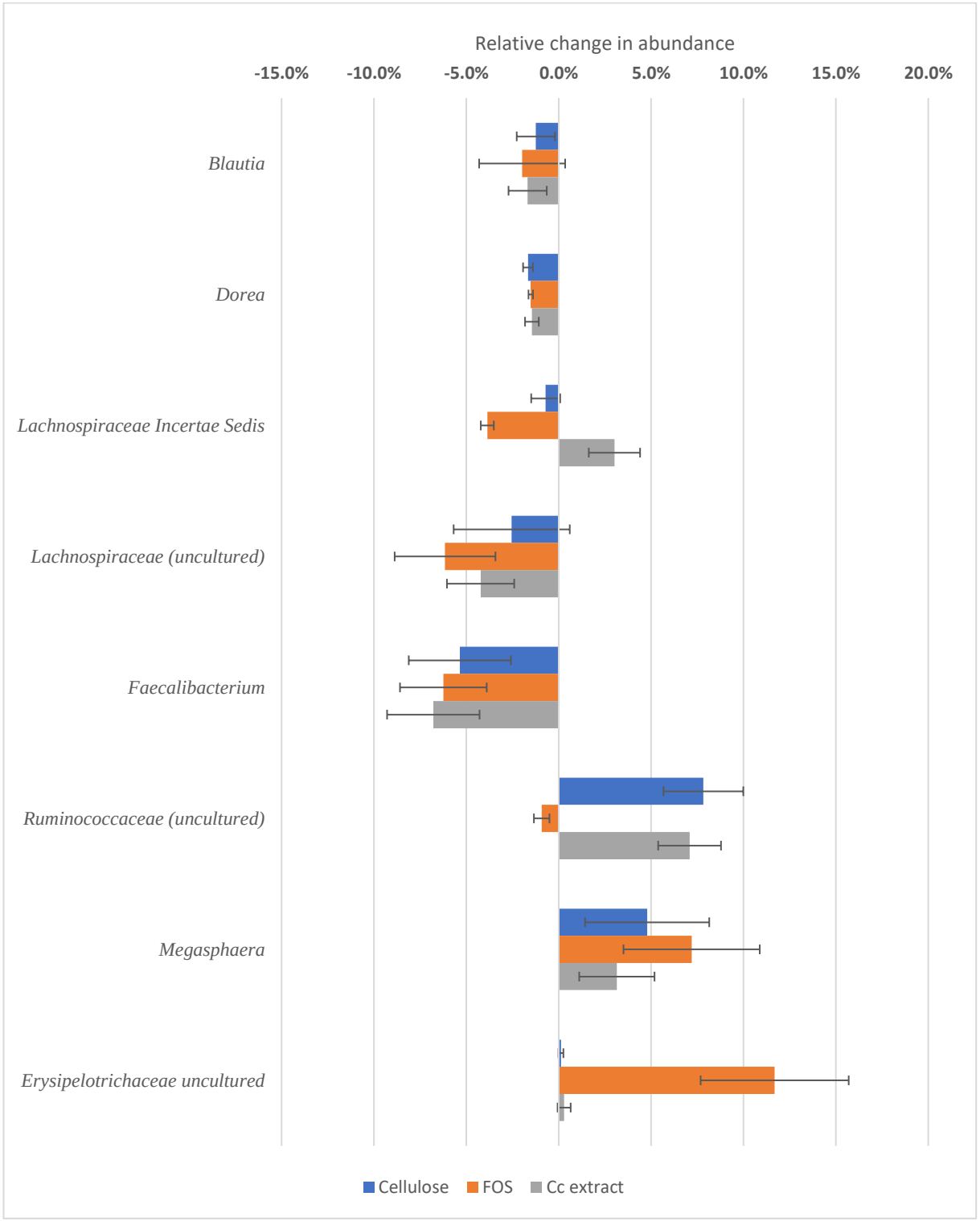
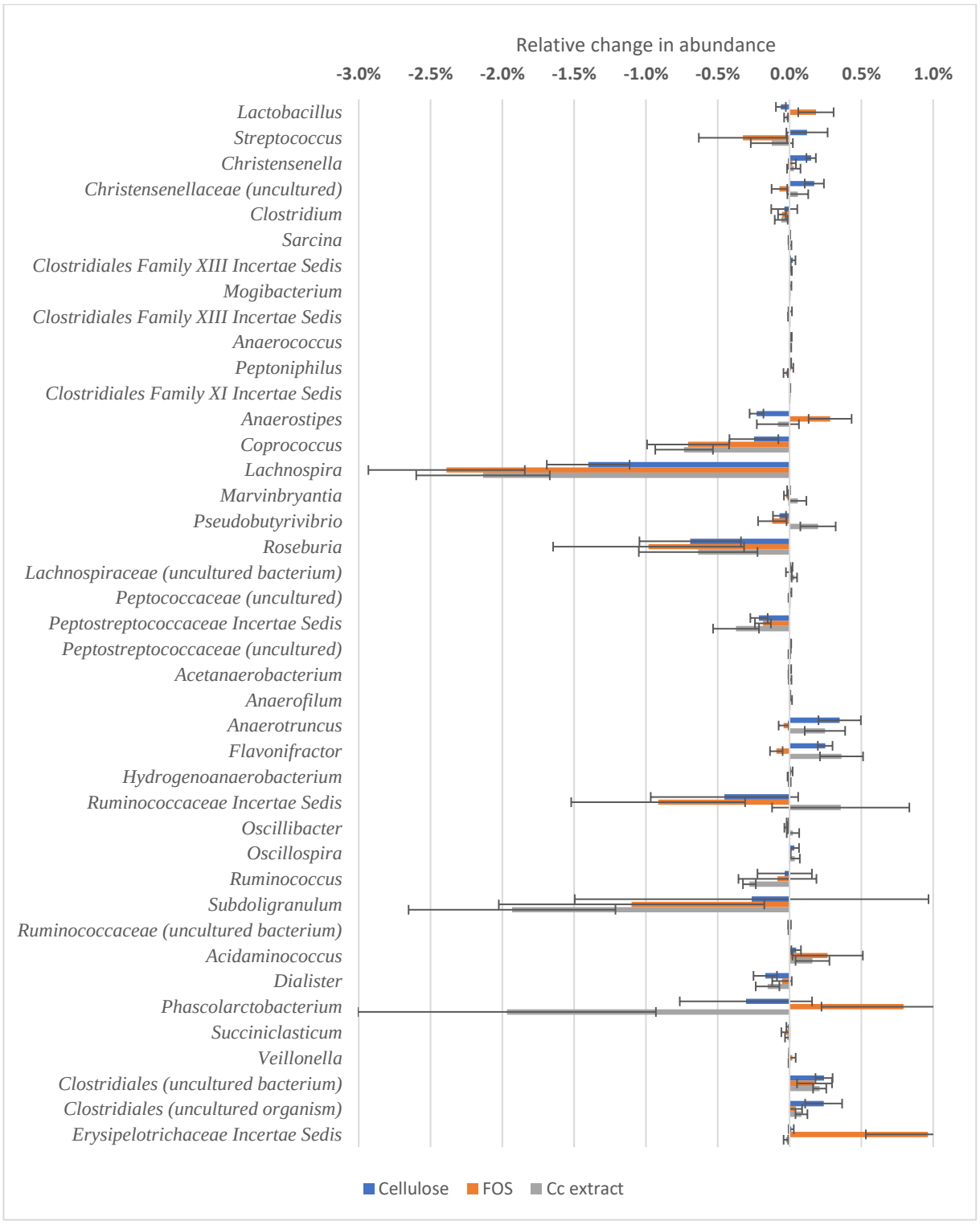

% change
-200
800
1800
2800
3800
4800
Methanobrevibacter
Actinomyces
Bifidobacterium
Gardnerella
Adlercreutzia
Collinsella
Eggerthella
Enterorhabdus
Coriobacteriaceae (uncultured)
Rhodococcus
Corynebacteriales (uncultured bacterium)
Arthrobacter
Bacteroides
Barnesiella
Butyricimonas
Odoribacter
Parabacteroides
Porphyromonas
Paraprevotella
Prevotella
Alistipes
Rikenellaceae RC9 gut group
Bacteroidales S24-7 (uncultured bacterium)
Bacteroidales S24-7 (uncultured organism)
Prevotellaceae (uncultured bacterium)
VC2.1 Bac22 (uncultured bacterium)
Fibrobacter
Victivallis
RF9 (uncultured bacterium)
vadinHA64 (uncultured bacterium)
Akkermansia
Cellulose
FOS
CC extract

Figure 5.12. (a) Increase/decrease in relative abundance of genera in the phylum Firmicutes. (b) Percentage change in the relative abundance of genera in the phylum Firmicutes. Data represent the mean (±SE).

(a)




Relative change in abundance
-3.0%
-2.5%
-2.0%
-1.5%
-1.0%
-0.5%
0.0%
0.5%
1.0%
Lactobacillus
Streptococcus
Christensenella
Christensenellaceae (uncultured)
Clostridium
Sarcina
Clostridiales Family XIII Incertae Sedis
Mogibacterium
Clostridiales Family XIII Incertae Sedis
Anaerococcus
Peptoniphilus
Clostridiales Family XI Incertae Sedis
Anaerostipes
Coprococcus
Lachnospira
Marvinbryantia
Pseudobutyrivibrio
Roseburia
Lachnospiraceae (uncultured bacterium)
Peptococcaceae (uncultured)
Peptostreptococcaceae Incertae Sedis
Peptostreptococcaceae (uncultured)
Acetanaerobacterium
Anaerofilum
Anaerotruncus
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Oscillospira
Ruminococcus
Subdoligranulum
Ruminococcaceae (uncultured bacterium)
Acidaminococcus
Dialister
Phascolarctobacterium
Succiniclasticum
Veillonella
Clostridiales (uncultured bacterium)
Clostridiales (uncultured organism)
Erysipelotrichaceae Incertae Sedis
Cellulose
FOS
Cc extract

(b)

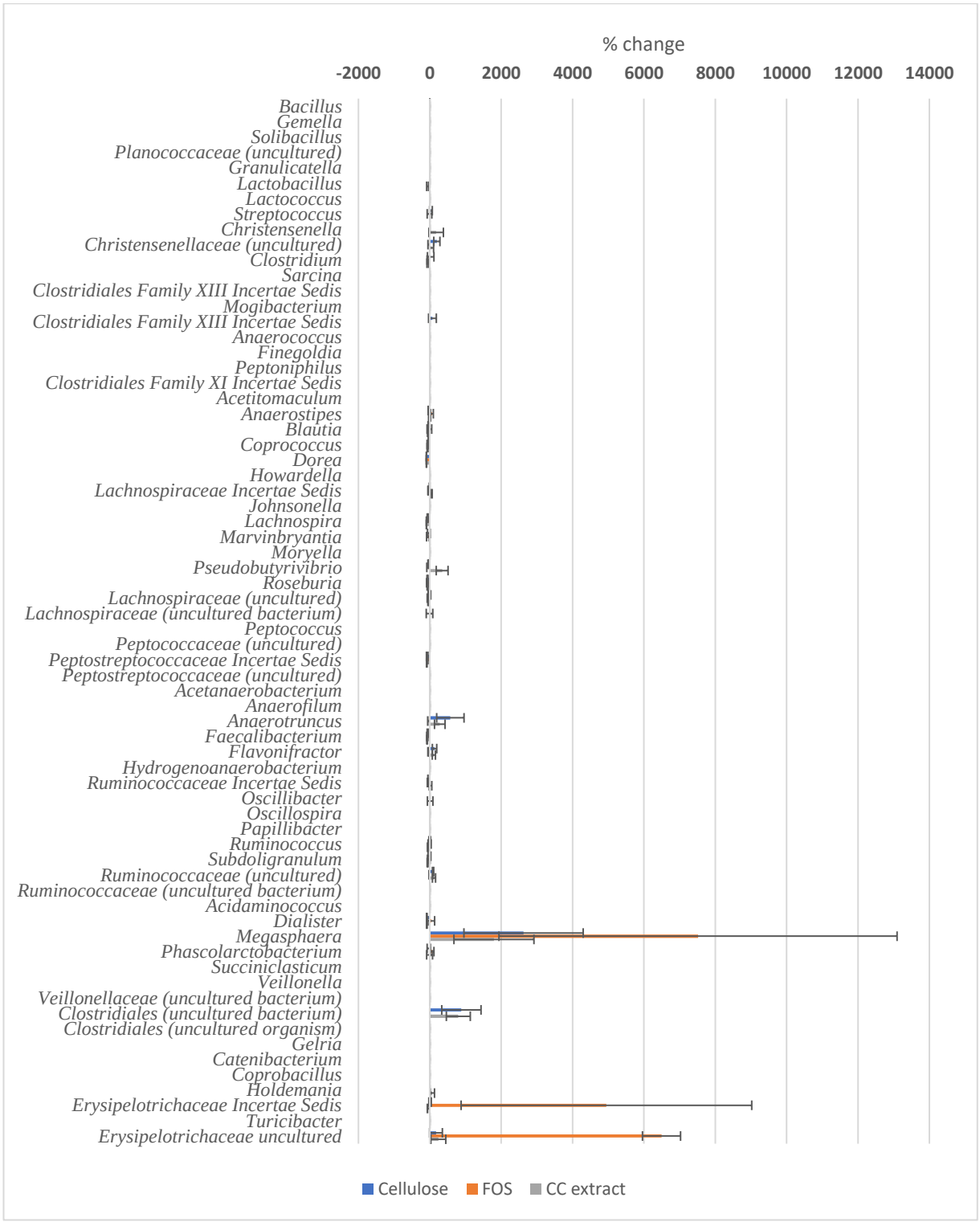
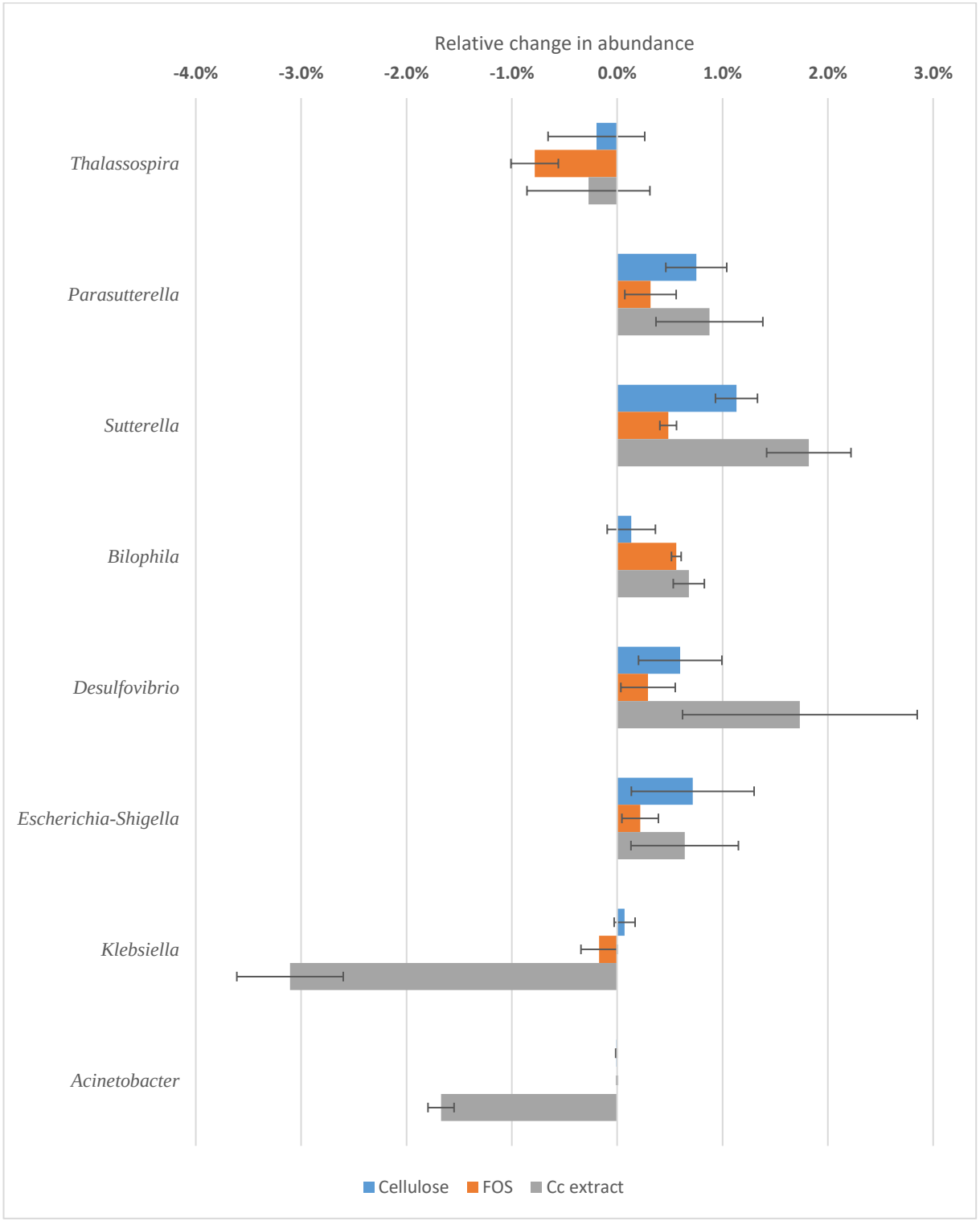
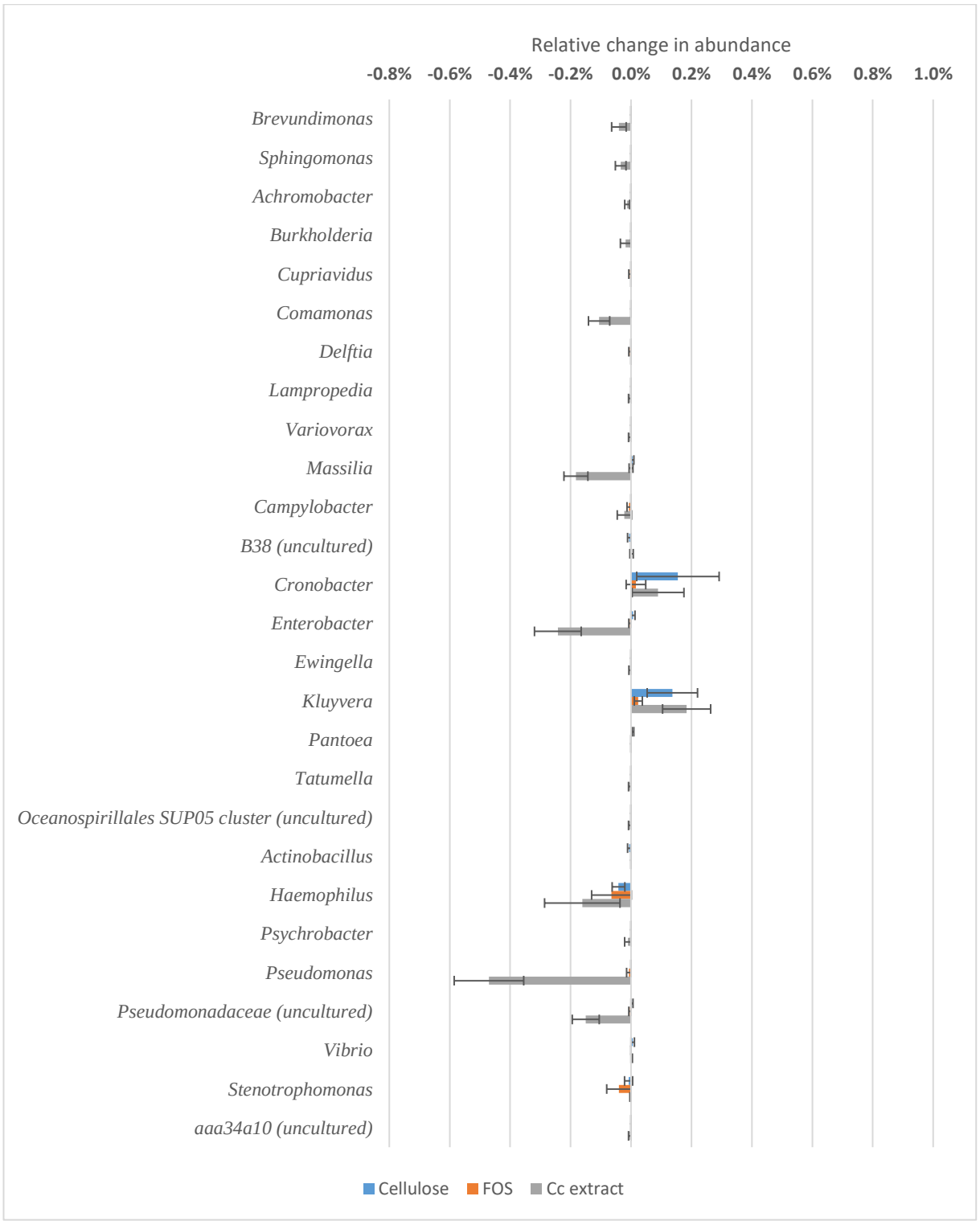

% change
-2000
0
2000
4000
6000
8000
10000
12000
14000
Bacillus
Gemella
Solibacillus
Planococcaceae (uncultured)
Granulicatella
Lactobacillus
Lactococcus
Streptococcus
Christensenella
Christensenellaceae (uncultured)
Clostridium
Sarcina
Clostridiales Family XIII Incertae Sedis
Mogibacterium
Clostridiales Family XIII Incertae Sedis
Anaerococcus
Finegoldia
Peptoniphilus
Clostridiales Family XI Incertae Sedis
Acetitomaculum
Anaerostipes
Blautia
Coprococcus
Dorea
Howardella
Lachnospiraceae Incertae Sedis
Johnsonella
Lachnospira
Marvinbryantia
Moryella
Pseudobutyrivibrio
Roseburia
Lachnospiraceae (uncultured)
Lachnospiraceae (uncultured bacterium)
Peptococcus
Peptococcaceae (uncultured)
Peptostreptococcaceae Incertae Sedis
Peptostreptococcaceae (uncultured)
Acetanaerobacterium
Anaerofilum
Anaerotruncus
Faecalibacterium
Flavonifractor
Hydrogenoanaerobacterium
Ruminococcaceae Incertae Sedis
Oscillibacter
Oscillospira
Papillibacter
Ruminococcus
Subdoligranulum
Ruminococcaceae (uncultured)
Ruminococcaceae (uncultured bacterium)
Acidaminococcus
Dialister
Megasphaera
Phascolarctobacterium
Succiniclasticum
Veillonella
Veillonellaceae (uncultured bacterium)
Clostridiales (uncultured bacterium)
Clostridiales (uncultured organism)
Gelria
Catenibacterium
Coprobacillus
Holdemania
Erysipelotrichaceae Incertae Sedis
Turicibacter
Erysipelotrichaceae uncultured
Cellulose
FOS
CC extract

Figure 5.13 (a) Increase/decrease in relative abundance of genera in the phylum Proteobacteria. Percentage change in the relative abundance of genera in the phylum Proteobacteria. Values represent the mean (±SE).

(a)




Relative change in abundance
-0.8%
-0.6%
-0.4%
-0.2%
0.0%
0.2%
0.4%
0.6%
0.8%
1.0%
Brevundimonas
Sphingomonas
Achromobacter
Burkholderia
Cupriavidus
Comamonas
Delftia
Lampropedia
Variovorax
Massilia
Campylobacter
B38 (uncultured)
Cronobacter
Enterobacter
Ewingella
Kluyvera
Pantoea
Tatumella
Oceanospirillales SUP05 cluster (uncultured)
Actinobacillus
Haemophilus
Psychrobacter
Pseudomonas
Pseudomonadaceae (uncultured)
Vibrio
Stenotrophomonas
aaa34a10 (uncultured)
Cellulose
FOS
Cc extract

(b)

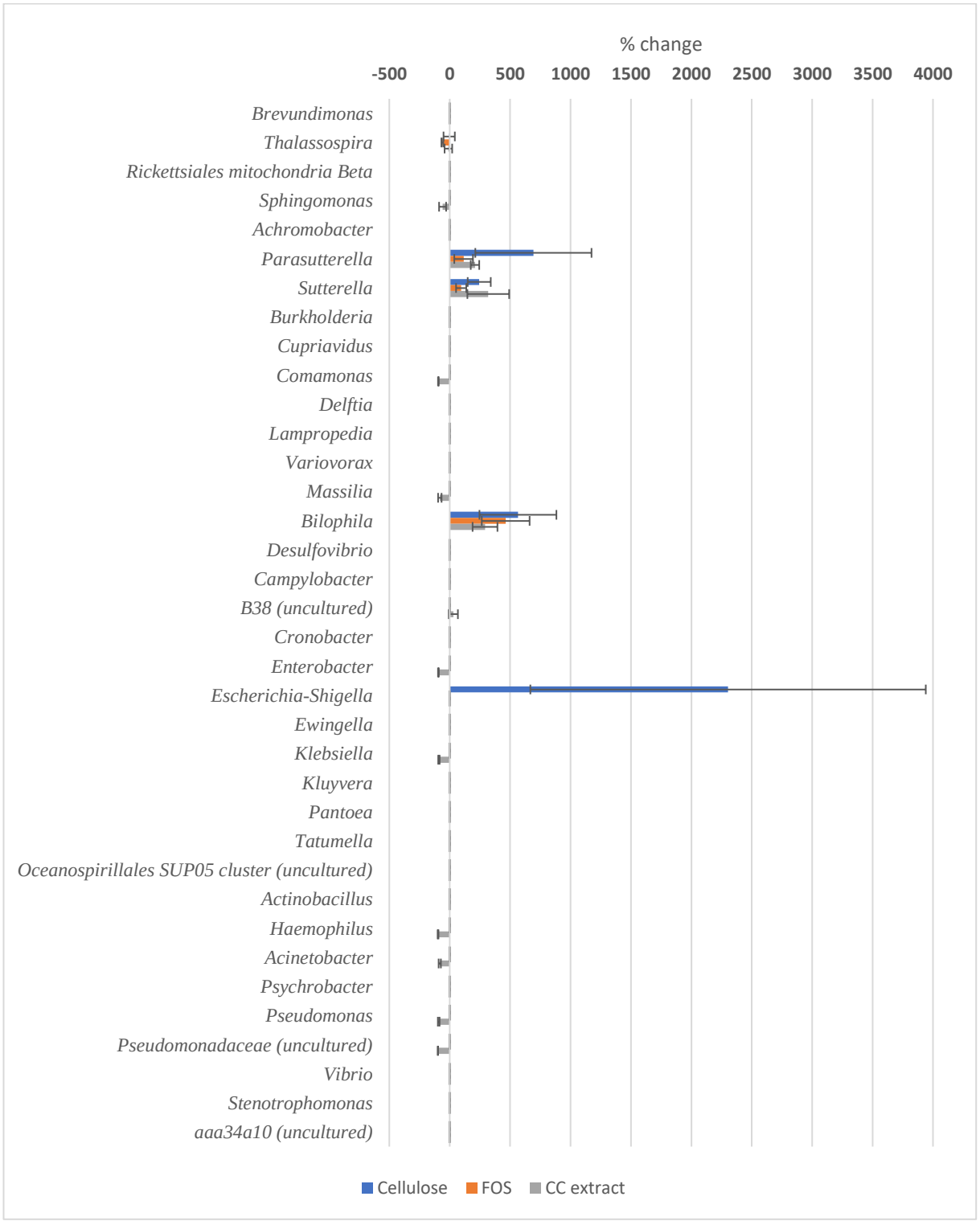

% change
-500
0
500
1000
1500
2000
2500
3000
3500
4000
Brevundimonas
Thalassospira
Rickettsiales mitochondria Beta
Sphingomonas
Achromobacter
Parasutterella
Sutterella
Burkholderia
Cupriavidus
Comamonas
Delftia
Lampropedia
Variovorax
Massilia
Bilophila
Desulfovibrio
Campylobacter
B38 (uncultured)
Cronobacter
Enterobacter
Escherichia-Shigella
Ewingella
Klebsiella
Kluyvera
Pantoea
Tatumella
Oceanospirillales SUP05 cluster (uncultured)
Actinobacillus
Haemophilus
Acinetobacter
Psychrobacter
Pseudomonas
Pseudomonadaceae (uncultured)
Vibrio
Stenotrophomonas
aaa34a10 (uncultured)
Cellulose
FOS
CC extract

Figure 5.15 Alpha diversity. (a) Chao1 richness estimation, (b) Shannon's index of diversity, (c) Simpson index of diversity, (d) Observed species and (e) Phylogenetic diversity metrics were used to estimate alpha diversity.

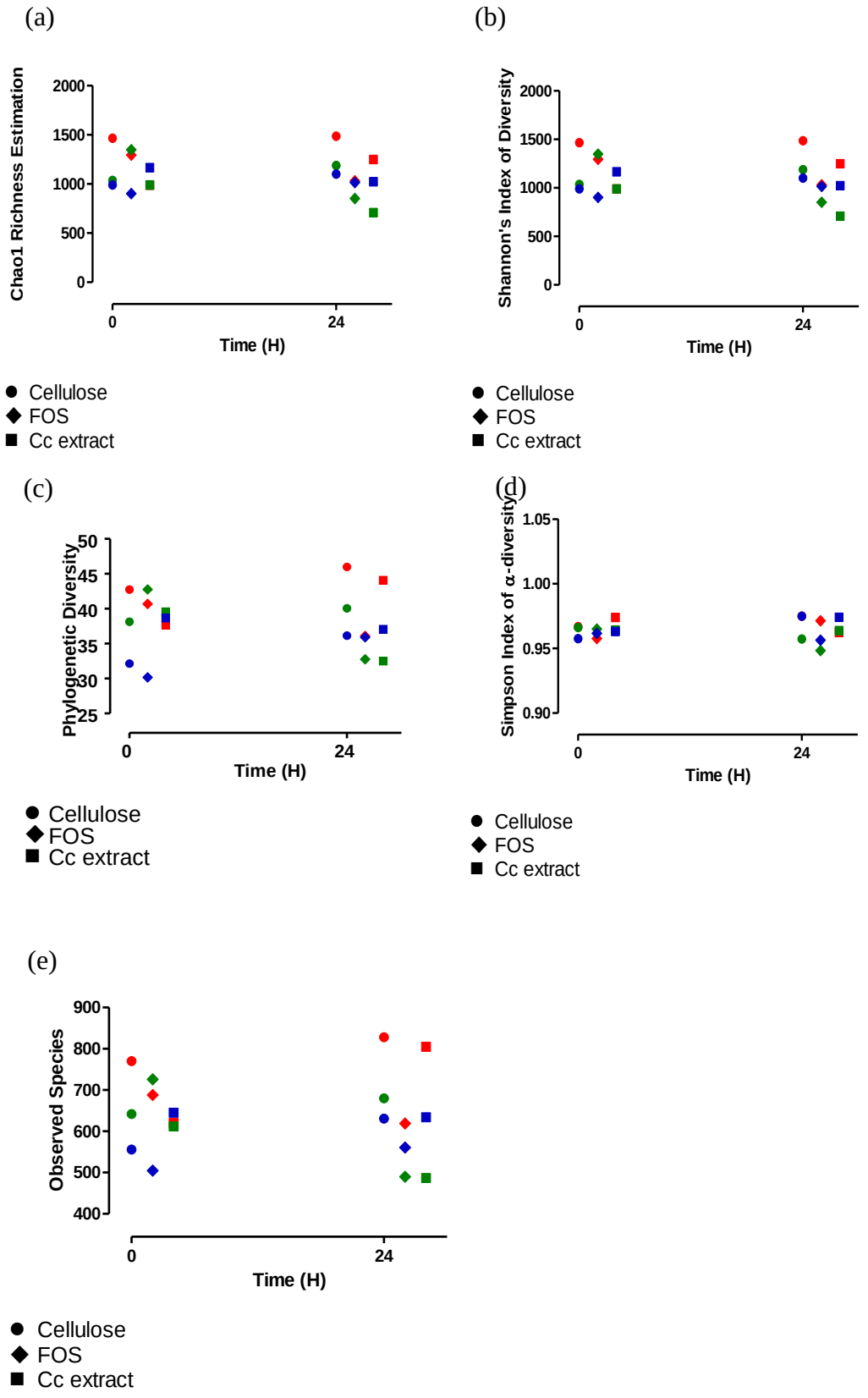
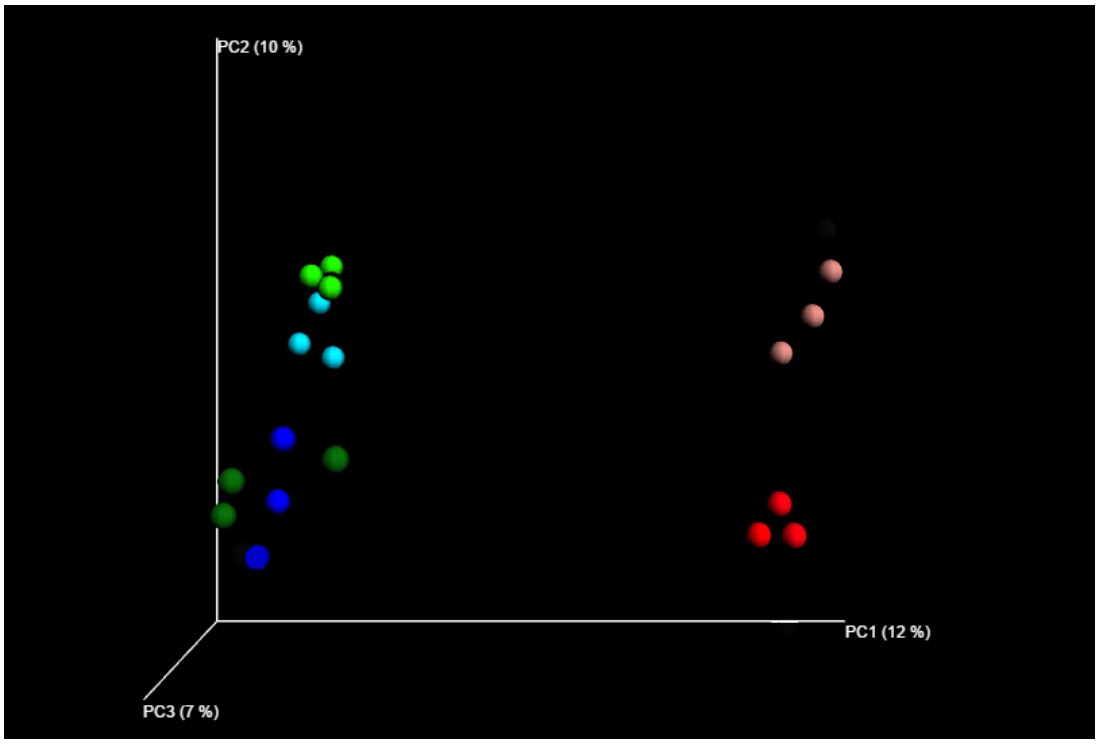


Figure 5.16 Beta diversity**.** Principle coordinate analysis of unweighted Unifrac reveals separation by fermentation run. Light Blue - Run 1 0 h, Light Green – Run 2 0 h, Light Red – Run 3 0 h, Dark Blue - Run 1 24 h, Dark Green – Run 2 24 h, Dark Red – Run 3 24 h.



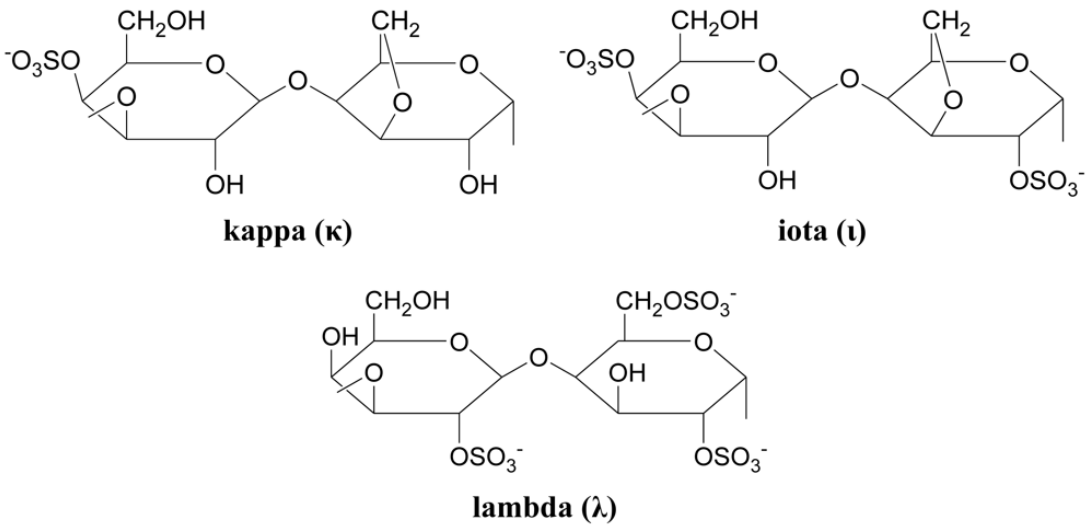
Supplementary Table 5.1 (a) To generate 16s rRNA bacterial gene amplicons (V4), a different version of the same forward primer was used for each fermentation sample. Each version contained a unique a distinct multiple identifier (MID) barcode allowing for distinction between the different samples. (b) A combination of four different reverse primers were used in conjunction with a single forward primer to generate16s rRNA bacterial gene amplicons.

Sample	Primer	Clamp	Barcode	Oligo
R1 AT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGAGA	AYTGGGYDTA
R1 BT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGAGA	AYTGGGYDTA
R1 DT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGAGC	AYTGGGYDTA
R1 AT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGATG	AYTGGGYDTA
R1 BT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGATG	AYTGGGYDTA
R1 DT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGCAG	AYTGGGYDTA
R2 AT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGCTC	AYTGGGYDTA
R2 BT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGCTC	AYTGGGYDTA
R2 DT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	AGCTG	AYTGGGYDTA
R2 AT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	ATCATC	AYTGGGYDTA
R2 BT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	ATCATC	AYTGGGYDTA
R2 DT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	ATCTCA	AYTGGGYDTA
R4 AT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	CAGAG	AYTGGGYDTA
R4 BT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	CTCAGC	AYTGGGYDTA
R4 DT0	Fusion45b	CCATCTCATCCCTGCGTGTCT	CAGAG	AYTGGGYDTA
R4 AT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	CAGCTC	AYTGGGYDTA
R4 BT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	CAGCTC	AYTGGGYDTA
R4 DT24	Fusion45b	CCATCTCATCCCTGCGTGTCT	CATCTG	AYTGGGYDTA

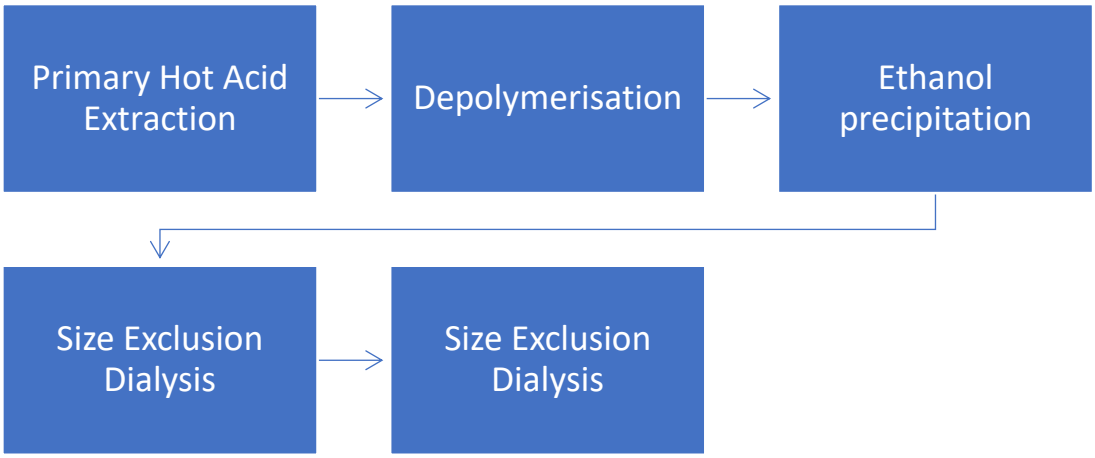
(b)

Primer name	Clamp	Oligo
Fusion45bc1L	CCTATCCCCTGTGTGCCTTGGCAGTC TCAG	TACNVGGGTATCTAATCC
Fusion45bc2L	CCTATCCCCTGTGTGCCTTGGCAGTC TCAG	CTACDSRGGTMTCTAATC
Fusion45bc4L	CCTATCCCCTGTGTGCCTTGGCAGTC TCAG	TACCAGAGTATCTAATTC
Fusion45bc9L	CCTATCCCCTGTGTGCCTTGGCAGTC TCAG	TACCRGGGTHTCTAATCC

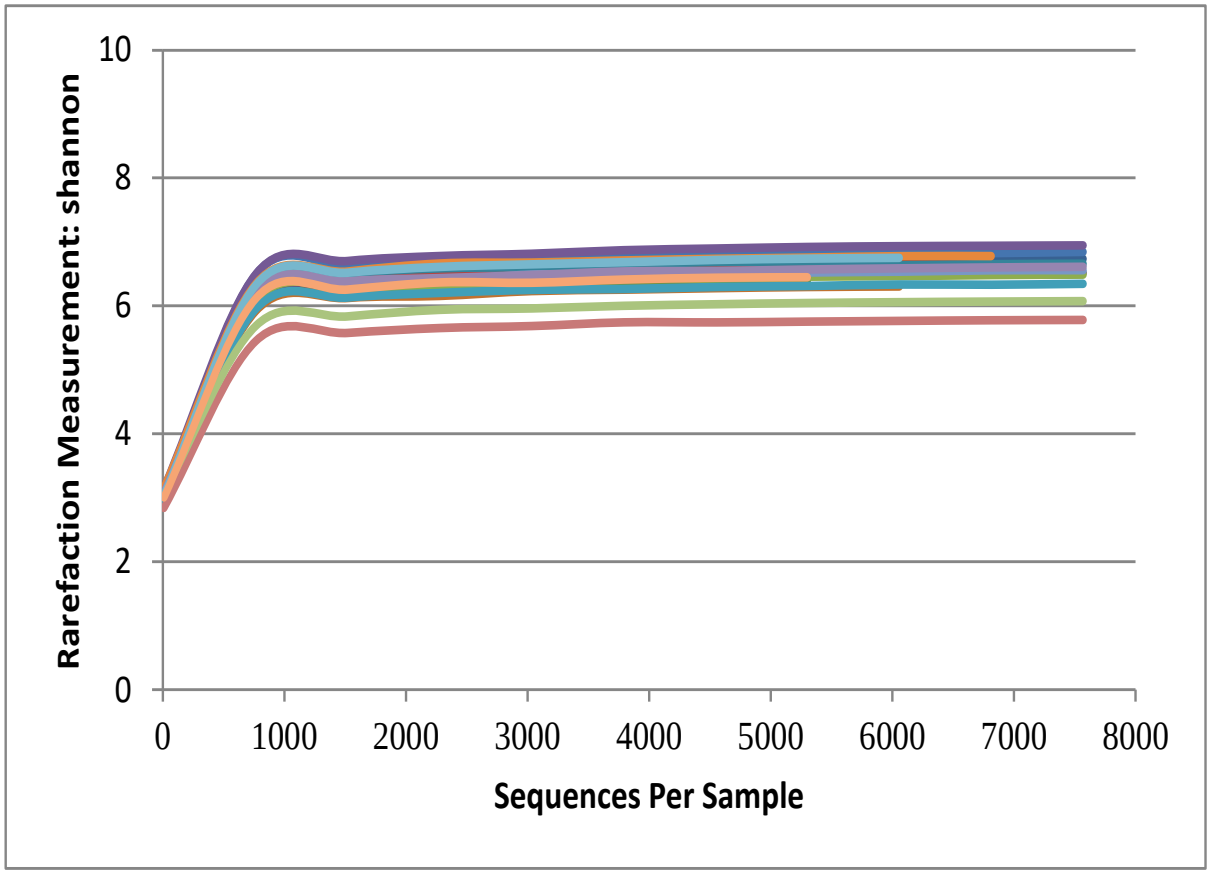
Supplementary Figure 5.1 Basic structure of kappa-, iota-, and lambda-carrageenan.



Supplementary Figure 5.2. Overview of the preparation of the C. crispus polysaccharide-rich extract.



Supplementary Figure 5.3 Rarefaction curve for each group at 97% similarity levels. Amount of operational taxonomic nits (OUT's) found as a function of the number of sequence tags sampled.



Supplementary Table 5.2 Alpha diversity. (a) Chao1 richness estimation, (b) Shannon's index of diversity, (c) Simpson index of diversity, (d) Observed species and (e) Phylogenetic diversity metrics were used to estimate alpha diversity.

Sample	Time-point	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
Control run 1	0 h	1,465.43	0.97	6.77	42.74	770.00
Control run 2	0 h	1,035.98	0.97	6.68	38.14	642.00
Control run 3	0 h	989.06	0.96	6.40	32.14	556.00
FOS run 1	0 h	1,295.62	0.96	6.54	40.69	688.00
FOS run 2	0 h	1,348.45	0.97	6.68	42.77	726.00
FOS run 3	0 h	903.48	0.96	6.31	30.18	505.00
Cc extract run 1	0 h	987.69	0.97	6.86	37.65	620.00
Cc extract run 2	0 h	990.28	0.96	6.59	39.58	612.00
Cc extract run 3	0 h	1,165.60	0.96	6.50	38.66	645.00
Control run 1	24 h	1,485.28	0.97	6.98	45.99	828.00
Control run 2	24 h	1,187.42	0.96	6.37	40.07	680.00
Control run 3	24 h	1,101.78	0.97	6.79	36.14	631.00
FOS run 1	24 h	1,031.50	0.97	6.57	36.09	619.00
FOS run 2	24 h	852.11	0.95	5.78	32.76	490.00
FOS run 3	24 h	1,014.92	0.96	6.08	35.91	561.00
Cc extract run 1	24 h	1,249.02	0.96	6.65	44.08	805.00
Cc extract run 2	24 h	707.57	0.96	6.46	32.49	487.00
Cc extract run 3	24 h	1,022.99	0.97	6.76	37.06	634.00

Chapter 6

The anti-microbial activity of Irish seaweed extracts against foodborne pathogens.

6.1 Abstract.

Foodborne illnesses are a major burden on health services throughout the world. A variety of antimicrobial agents have been used to prolong the shelf-life of processed goods and inhibit the growth of foodborne pathogens but resistance to traditional antimicrobials is spreading quickly. *Listeria monocytogenes* is among the most prevalent foodborne pathogen and is the causative agent of listeriosis. This organism can tolerate an extensive range of environmental conditions - such as refrigeration temperatures, and survives well under both aerobic and anaerobic conditions. These attributes combine to make *Listeria monocytogenes* a major concern in the production of Ready-to-Eat (RTE) foods. In this study, ten ethanol extractions derived from Irish brown seaweeds, were examined for their antimicrobial activity against a selection of foodborne pathogens including *Listeria monocytogenes*. Extracts from the seaweeds *Fucus vesiculosus, F. serratus F. spiralis, Ascophyllum nodosum* and *Pelvetia canaliculata* significantly inhibited ($p<0.05$) the growth of *Listeria monocytogenes* 5788 at 24 h. The *F. vesiculosus* extract was chosen for further evaluation. Molecular weight fractions of *F. vesiculosus* were tested against several Listeria stains. It was found that the anti-listerial activity was concentrated in the 0 - 3.5 kDa and the 3.5-100 kDa molecular weight subfractions, with the 3.5 - 100 kDa exhibiting the highest activity of all. Antimicrobial activity was found to be positively correlated with elevated levels of phenolic compounds. *F. vesiculosus* was found to have the highest level of phenolic compounds amongst all the seaweed species tested at 138.3 ± 0.7 µg GAE mg^{-1} and demonstrated the most potent activity against the pathogen *L. monocytogenes*. This is a promising outcome as to the potential of utilising such extracts from seaweeds in food products to serve as both biopreservation and antioxidants agents.

6.2 Introduction

Food related illness is a common and often preventable malaise [1] that affects approximately 30% of individuals living in industrialised countries every year. As such, it is a major burden on health services throughout the world [2]. The Centre for Disease Control (CDC) in Atlanta projects that each year in the US alone more than 5,000 people die of foodborne related illnesses. [3]. World-wide, six pathogens alone are estimated to account for over 90% of food-related deaths: *Salmonella*, *Listeria*, Toxoplasma, Norwalk-like viruses, *Campylobacter*, and *Escherichia coli* O157:H7 [3]. Among the most important avenues of treatment of bacterial infection today are antibiotics. Antibiotics are organic or synthetic compounds that even at a low level have deleterious effects on the growth and/or metabolism of susceptible microorganisms. They have been in large scale use since the development of the sulphonamides in the 1930's. However, current antibiotics have been associated with several negative traits such as: high-cost, toxicity, residual side effects, allergic reactions, hypersensitivity and immunosuppression. The over reliance on antibiotics and their misuse by health care workers and the public alike have also resulted in the emergence of antibiotic-resistant bacterial strains, leading to a reduction in effectiveness of anti-microbial therapy in some instances. The WHO has classified the spread of antibiotic resistance among bacteria as one of the biggest threats to public health in the 21st century. Because the extensive use of antibiotics can alter the normal human gut microbiota, enrichment with antibiotic resistant bacteria which further complicates treatment, can occur [4, 5]. Many naturally-occurring compounds found in edible and medicinal plants, herbs and spices have been shown to possess antimicrobial properties [6] but the search for new antimicrobials to date has mainly focused on terrestrial environments. Algae are a proven source of novel bioactive compounds [2]. In this respect the long evolution of marine plants, compared with their terrestrial counterparts, has resulted in the generation of a huge diversity of genes, species etc. This diversity, coupled with the ability of plants to adapt, compete and survive in extreme environmental conditions, has made marine organisms potentially a very valuable source for novel antimicrobial activities. Moreover, they possess the ability to synthesise a variety of unique chemical structures, many of which have

potent bioactive/antimicrobial activity [7]. The rise of resistance to antibiotics makes the development of novel strategies to prevent and treat bacterial infections crucial.

Seaweeds are multicellular photosynthetic organisms that are found in abundance in both shore and offshore areas [8]. Based on the composition of their photosynthetic pigments, they are generally classified in to three groupings; the Phaeophyta (brown seaweeds), the Chlorophyta (green seaweeds), and the Rhodophyta (red seaweeds) [9]. Close to 10,000 different species of seaweed have been catalogued so far [10]. Both fresh and dry seaweeds are traditionally consumed as vegetables in countries of East Asia most notably Japan, China and Korea. Edible species of seaweed have a high nutritional value with proteins and lipids that are of a comparatively better quality than other terrestrial vegetables. Seaweeds are also used for animal feed applications and in soil cultivation as a fertilizer [4]. Seaweeds are a great source of structurally novel secondary metabolites [10] that are postulated to play an important role in mediating marine host-microbe interactions in the ocean [11]. Secondary metabolites are produced as a chemical defense against the biotic pressure placed on them by predators, consumers and epibionts as well as the abiotic pressure of the immediate environment (e.g. nutrient deficiency, desiccation, UV) which can affect their growth. Potential drugs from seaweeds are now attracting a considerable amount of attention from the pharmaceutical sector due to the ever-growing need to find new substances with antimicrobial activity against problematic human pathogens. The therapeutic benefits of seaweeds in traditional medicines have been known for centuries, but it was only at the beginning of the 20th century that antimicrobial substances produced by algae were observed. Today it is known that seaweed derived compounds have a broad range of biological actions such as, antibiotic, anticoagulant, antioxidant, antiviral, antineoplastic, antifouling, anti-inflammatory, anti-mitotic, anti-thrombotic, anti-lipemic, anti-cytotoxic, anti-hypotensive, anti-ichthyotoxic and anti-mitogenic activities [12]. These beneficial properties are contributed by the various types of carotenoids and polyphenols present in seaweeds - among the most prominent kinds are catechin, phlorotannins, fucoxanthin, zeaxanthin, β-carotene, and lutein [13]. Other compounds synthesized by seaweeds include acetogenins, alkaloids, lipids, phenolic compounds, pigments, polysaccharides, proteins, polyunsaturated fatty acids, and terpenes [14]. Many seaweed species have also been shown to produce substances that demonstrate bacteriostatic or bacteriocidal activity [15]. The

bacteriocidal compounds belong to various chemical groups such as terpenoids, phlorotannins and other phenolic compounds, acrylic acid, steroids, halogenated ketones and alkanes, cyclic polysulphides, amino acids and fatty acids. The production of antimicrobial substances by seaweed species can vary for different reasons such as environmental factors, season, geographic location, the stage of life cycle as well as different parts of the thallus [12, 14]. In general, these compounds can (a) target the bacterial cell wall and membrane, which results in an extensive release of intracellular substances and/or disruption of the uptake and transportation of substances (e.g. phlorotannins) or (b) reduce protein and nucleic acid synthesis or (c) inhibit respiration. Phlorotannins may also form complexes with some extracellular bacterial enzymes, thus reducing their activity. Bioactive substances from seaweeds, such as, furanones, can also interfere with quorum sensing mechanisms in bacteria which can affect their virulence [16]. Phenolic compounds possess great structural variations and are one of the most diverse groups of secondary metabolites. The hydroxyl groups in phenolic compounds are thought to interfere with membrane structure causing leakage of cellular components [17]. Interestingly, extracts from seaweeds have been found to be more active against Gram-positive bacteria than Gram-negative bacteria which may be related to the other membrane functioning as an extra barrier [35].

A major concern in the production of ready-to-eat (RTE) foods is the contamination of goods by the Gram-positive, facultative anaerobic bacterium *Listeria monocytogenes* [18]. *L. monocytogenes* is a pathogen of both humans and animals, and has also been isolated from a variety of raw dairy, meat, and meat products, sea foods and fresh products [19]. It is also the etiologic agent of listeriosis which is an extremely serious foodborne disease that can be life threatening to individuals in high risk categories such as: the elderly, pregnant women, neonates and immune-compromised individuals [20]. Listeriosis is primarily acquired through the consumption of contaminated food. The first identified foodborne outbreak of Listeriosis occurred in the early 1980s and since then *L. monocytogenes* has become increasingly important as a foodborne pathogen and also a model system for infection biology [21]. The pathogenicity of *L. monocytogenes* is due to its capacity to adhere to, invade and multiply within a great variety of normally nonphagocytic cells (enterocytes, hepatocytes, fibroblasts, endothelial cells and dendritic cells)[22]. *L. monocytogenes* is also able to survive over a wide range of environmental conditions

having the capability to grow at refrigeration temperatures (2 - 4 °C), at pH ranging from 4.0 to 9.6 (optimum pH 6-8) and at water activity levels of 0.90 [23]. Additionally, *L. monocytogenes* can adhere to a variety of food contact surfaces such as stainless steel and polystyrene, and can persevere in food processing facilities for several months or even years as biofilms. Protected in biofilms, this organism can tolerate high concentrations of many environmental agents such as sanitizers, disinfectants and antimicrobial compounds, making it very difficult to eliminate effectively. The fact that *Listeria* can survive so many environmental challenges makes it a major concern for the food industry and public health services. RTE foods such as fish products, heat-treated meat products and cheese are often cited as sources of *Listeria* infection [23]. Antibiotic treatment of listeriosis has proven to be successful, with treatment usually encompassing the use of β-lactam antibiotics such as ampicillin or penicillin alone or in combination with an aminoglycoside (gentamicin). For those allergic to β-lactams, trimethoprim and sulphonamides have been successfully used as alternatives [19]. *Listeria* were considered relatively susceptible to a wide range of antibiotics until the discovery of an antibiotic resistant strain of *L. monocytogenes* in 1988 [24].

Current antibiotic treatments are often accompanied with undesirable aspects such as high expense, and toxicity; and residual side effects such as allergic reactions, hypersensitivity and immunosuppression. Researchers are now increasingly investigating alternative antimicrobial compounds from terrestrial plants and marine organisms such as seaweed [4]. The aim of this study was to access the antimicrobial activity of ten ethanol extracts prepared from ten brown seaweeds collected from along the Irish coast against a selection of pathogenic bacteria. Emphasis was given to the activity of different fractions of *Fucus vesiculosus* (Phaeophyceae) against the major food borne pathogen *Listeria monocytogenes*.

6.3 Materials and methods

6.3.1 Media.

All media used in this study was obtained from Sigma-Aldrich, Dublin, Ireland, unless otherwise stated. All plastic consumables were obtained from Sarstedt, Wexford, Ireland

6.3.2 Bacterial strains.

The bacterial strains used in this study were obtained from the Teagasc Research Facility, Moorepark culture collection and were as follows: *Listeria monocytogenes* 5788, *L. monocytogenes EGDe*, *L. monocytogenes* DPC 4590 ATC 19112 and *L. monocytogenes* DPC 4608 SLCC 1694; *Staphylococcus aureus* 5246; *Escherichia coli* O157:H7, *E. coli* 6235; and *Salmonella enterica* serovar Typhimurium DT104 (104-25). Strains were grown overnight on BHI agar (Merck, Darmstadt, Germany), or in BHI broth at 37 °C. Stocks of all strains were kept at - 80 °C with working stocks being stored at - 20 °C.

6.3.3 Seaweed collection.

The seaweed samples used in this study were sourced from four collection points along the western coast of Ireland; Finnavara, Co. Clare (F), Mweenish, Co. Galway (M), New Quay, Co. Clare (NQ) and Spiddal, Co. Galway (S). Harvesting took place between April 2009 and February 2011. The species of seaweed used in the study were as follows; *Alaria esculenta* (Phaeophyceae) (S), *Laminaria digitata* (Phaeophyceae) (S) (May 2009), *Himanthalia elongata* (Phaeophyceae) (F) (April 2010), *Saccharina latissima*, (Phaeophyceae) (S) (September 2010), *Fucus serratus* (Phaeophyceae) (S), *Fucus vesiculosus* (Phaeophyceae) (S) and *Ascophyllum nodosum* (Phaeophyceae) (NQ) (October 2010), *Pelvetia canaliculata* (Phaeophyceae) (S), *Chondrus crispus* (Rhodophyta) (F), *Fucus spiralis* (Phaeophyceae) (S), and *Laminaria hyperborea* (Phaeophyceae) (M) (February, 2011).

6.3.4 Preparation of ethanol 80% extracts and fractions for antimicrobial study.

Following collection, the seaweeds were washed with cold water to remove particulate matter. Samples were freeze-dried, ground to a fine powder, vacuum packed and stored at -80 °C prior to extraction. A solvent liquid extraction using ethanol/water (80:20) as the solvent was then carried out. Dried seaweed matter was mixed in a ratio of 1:10 (w/v) with the extraction solvent in a large reaction vessel and placed in an orbital shaker (MaxQ 6000 shaker, Thermo Fisher Scientific, Ireland) at 175 rpm. After 3 h, the reaction vessel was removed from the orbital shaker and its contents were filtered through a Buchner funnel. The seaweed material was then returned to the reaction vessel with fresh extraction solvent and the process repeated. The extraction was repeated a third time overnight to ensure complete extraction from the seaweed material. The collected solvents were then combined, and the ethanol removed using a rotary evaporator with the water bath set at 60 °C. The extracts were then blast-frozen and freeze-dried and stored at -80 °C as a fine powder. The sugar fraction was obtained by ethanol precipitation using 100% ethanol at a ratio of seaweed: ethanol of 1:5. The mixture was then centrifuged for 5 min at 5000 g and the precipitate pellet was collected. Fresh deionised water was finally used to resuspend the pellet prior to being blast and freeze-dried.

Fractions of *F. vesiculosus* (0 - 3.5 kDa, 3.5 - 100 kDa, and > 100 kDa) were prepared as follows: the crude ethanol extract was dissolved in a minimal volume of deionised water and transferred to 3.5 kDa cut-off dialysis tubing (Spectrum labs, Breda, The Netherlands). The tubing was clamped at both ends and submerged in deionised water. The container was then moderately shaken (50 rpm) at room temperature for 72 h. The water in the container was routinely replaced with fresh deionised water until the dialysate ran colourless. Both the higher molecular weight retentate (> 3.5 kDa) and the low molecular weight dialysate (0 - 3.5 kDa) were collected and subsequently freeze-dried. The retentate greater than 3.5 kDa was further dissolved in a minimal volume of deionised water and added to 100 kDa dialysis tubing. Both the retentate and dialysate were then freeze-dried.

6.3.5 Microtitre plate-based dilution method for antimicrobial activity.

Each seaweed extract was prepared in 10 mM sodium phosphate buffer with 2% vol/vol DMSO being added in to aid in solubility. The stock concentration of extract was 2 mg/ml with a working concentration of 1 mg/ml. The extract was filter sterilized using a 0.45 µm filter. Bacterial strains were grown in triplicate overnight in 5 ml BHI broth at 37 °C and subcultured (1 ml of culture added to 5 ml BHI broth) the following morning in fresh media to an OD_{600nm} of 0.3. Cells were harvested from 1 ml samples of subcultured cells by centrifugation at 15000 g for 5 min. The supernatant was aspirated and the bacterial pellets were washed twice and resuspended in 1 ml of sodium phosphate buffer. Serial dilutions were then carried out in sodium phosphate buffer for plate counting. Fifty microliters of the stock seaweed extract were added to 50 µl of cells (1:100 dilutions) in a 96-well microtitre plate. The plate was incubated at 37 °C for 2 h without media as some media components can inhibit the activity of antimicrobial peptides Following the incubation step, 100 µl of double strength BHI broth was added to each well to allow the outgrowth of surviving bacteria. The plate was then transferred to a microplate reader where readings of OD_{600nm} were taken every hour for 24 h. Percentage inhibition was calculated according to the equation.

$$\text{Inhibition (\%)} = \frac{O - E}{0} \text{ X } 100$$

Where O is (OD_{600nm} of the organism at 24 h - OD_{600nm} of the organism at 0 h) and E is (OD_{600nm} of the organism in the presence of the seaweed extract at 24 h – blank at 24 h) – (OD_{600nm} of the organism in the presence of the extract at 0 h – the blank at 0 h) [25].

6.3.6 Total phenolic content analysis of seaweed extracts.

The total phenolic content (TPC) of the seaweed extracts was assessed using previously described methods. Gallic acid (Sigma – Aldrich) was used as the phenol

external standard for the quantification of phenolic compounds in the seaweed samples. A stock solution of gallic acid (120 µg/mL) was serially diluted with water (120, 90, 60, 30, and 0 µg/mL) to generate a standard curve. To analyse each sample for TPC, the following were added to a microtube: 100 µl of seaweed sample (1 mg/mL) or standard solution, 100 µl of methanol, 100 µl of Folin-Ciolalteu reagent (Sigma – Aldrich) and 700 µl of 20% (m/v) sodium carbonate. The tubes were then vortexed vigorously and incubated in the dark for 20 min at room temperature. Following incubation, all samples were centrifuged for 3 min at 12500 g. The absorbance of the supernatant taken from each sample was measured in triplicate at 735 nm using a spectrophotometer. The standard curve generated using the gallic acid standards was used to obtain the TPC values for the seaweed samples which are expressed as micrograms of gallic acid equivalents (GAE) per milligram of sample (µg GAE mg^{-1}) [26].

6.3.7 Statistical analysis.

Statistical analysis was carried out on experimental results using GraphPad Prism version 5 for windows. Percentage inhibition data was subjected to a one-way ANOVA followed by Dunnett's Multiple Comparison test. All experiments were carried out in duplicate with at least three biological repeats. A p-value of <0.05 was deemed to be a significant result.

6.4 Result

6.4.1 Antimicrobial activity of ten seaweed extracts against a selection of foodborne pathogenic bacteria.

Extracts derived from ten brown seaweeds were screened for antimicrobial activity against strains of Gram-positive (*L. monocytogenes* and *S. aureus)* and Gram-negative (*E. coli* and *S. enterica* serovar Typhimurium*)* pathogens. The positive controls used were Nisin from *Lactococcus lactis* (2.5% w/w) for Gram-positive organisms and Tetracycline for Gram-negative organisms. At a working concentration of 1 mg/ml, three seaweeds of the genus *Fucus* (*F. vesiculosus*, *F. serratus* and *F. spiralis*) (Fig. 6.1) and the seaweeds *A. nodosum*, *H. elongata*, *P. canaliculata* significantly inhibited ($p<0.05$) growth of *L. monocytogenes* 5788 at 24 h (Fig. 6.2,). The percentage inhibition of *Listeria monocytogenes* 5788 growth as measured by optical density was for *A. nodosum* (91.5% ± 12.4), *F. serratus* (91.8% ± 1.2), *F. spiralis* (94.0% ± 8.9), *F. vesiculosus* (complete inhibition), *H. elongata* (45.5% ± 10.8) and *P. canaliculata* (82.8% ± 16.5) (Fig. 6.3). No viable colony forming units were recovered at a 10^0 dilution on BHI agar plates for *A. nodosum*, *P. canaliculata* and all three *Fucus* species. The *F. vesiculosus* extract also significantly inhibited ($p>0.05$) the growth of *E. coli* 6235 after 24 h (Fig. 6.1) with a percentage inhibition (as measured by optical density readings) of 36.0% ± 5.9 (Fig. 6.3) Significant inhibition ($p<0.05$) of *S. aureus 5246* at 24 h by *A. nodosum*, *H. elongata* and *P. canaliculata* extracts and *E. coli* 6236 by *A. nodosum* (Fig. 6.2) and *F. vesiculosus* extracts was also observed (Fig 6.1).

6.4.2 Strain specificity of the *F. vesiculosus* ethanol extract.

The ETOH 80% extract of *F. vesiculosus* (Fv extract) was chosen for further evaluation. The Fv extract was tested for antimicrobial activity against three further strains of *L. monocytogenes* (4590, 4608 and EDGe) for antimicrobial activity (Fig. 6.4). At a concentration of 1000 µg/ml and 250 µg/ml, the Fv extract showed significant inhibitory ($p<0.05$) activity against all strains following at 24 h. At 1000 µg/ml, the percentage inhibition of each strain at 24 h was greater than 90% (Fig. 6.6).

At a dilution of 10^0 no viable CFUs were recovered from any of the tested strains on BHI agar plates after 24 h.

6.4.3 Anti-listeria activity of *F. vesiculosus* ethanol subfractions.

Different molecular weight fraction of the *F. vesiculosus* ethanol extract were evaluated for antimicrobial activity against two *Listeria* strains (*L. monocytogenes* 5788 and *L. monocytogenes* EGDe) as outlined previously (Fig. 6.7). The subfractions were 0 - 3.5 kDa, 3.5 - 100 kDa, 100 + kDa, tannins and sugars. The most potent and consistent anti-listerial activity was observed with the 0 - 3.5 kDa and the 3.5 - 100 kDa fractions. Both subfractions significantly inhibited ($p>0.0001$) the growth of *L. monocytogenes* 5788 and *L. monocytogenes* EGDe after 24 h. Plate counts at 24 h yielded no viable CFUs on BHI plates after 24 h with either test strain. The sugar fraction from *F. vesiculosus* had no inhibitory effect on the growth of any strain tested. Both fractions (0 – 3.5 kDa and 3.5 – 100 kDa) were further tested against *L. monocytogenes* 5788 (Fig. 6.8) at working concentrations of 1000 µg/ml, 100 µg/ml and 10 µg/ml. The 0 - 3.5 kDa and 3.5 - 100 kDa subfractions significantly inhibited ($p>0.05$) growth of *L monocytogenes* 5788 at 100 µg/ml, however, only the 3.5 - 100 kDa subfraction significantly inhibited growth at 10 µg/ml.

The 3.5 – 100 kDa extract was subsequently tested against a range of *Listeria* strains (5788, 4590, 4606 and EDGe) (Fig. 6.9) at working concentrations of 1000 µg/ml, 100 µg/ml and 10 µg/ml. Percentage inhibition of growth was about 80% for strains 4590, 4608 and EDGe at 100 µg/ml (Fig. 6.10). There was no recovery of viable CFUs at 24 h for any 100 µg/ml for strains 5788, 4608, EDGe. The complete inhibition of bacterial growth was observed for the nisin control at 1 mg/ml for all strains. Below this concentration, there was no significant inhibition of any of the test strains at 24 h (Fig 6.10)

6.4.4 Antimicrobial activity of the *F. vesiculosus* ethanol extracts against *S. aureus.*

The crude Fv extract and the 3.5 - 100 kDa fraction were tested for antimicrobial activity against two additional strains of the Gram-positive bacteria *S. aureus* (*S. aureus* strains FRI913 and Sa45). After 16 h, *Staphylococcus aureus* FRI913 growth was significantly reduced ($p<0.05$) by the *Fv* 3.5 - 100 kDa fraction with a percentage inhibition of 83.6% ± 2.7. The *Fv* crude extract had a minimal effect on growth after 16 h. The growth of *S. aureus* Sa45 after 1 h was significantly reduced ($p<0.05$) by the *Fv* crude extract with a percentage inhibition of 82.9% ± 20.1. The *Fv* 3.5 - 100 kDa fraction inhibited growth by 66.0% ± 46.7 Plate counts for surviving bacteria recorded no recovery of viable CFUs of either strain at a 10^0 dilution following testing of the 3.5 - 100kDa fraction.

6.4.5 Total phenolic content analysis of seaweed extract and Fv fractions

Given that phenolic compounds generally have potent antimicrobial activity, the total phenolic content of each seaweed extract and fraction was determined. Total phenolic content (TPC) values are reported in terms of micrograms of gallic acid equivalents (GAE) per milligram of sample (µg GAE mg^{-1}). The highest levels of phenolic compounds in unfiltered extracts were observed in the *F. vesiculosus* extract with 138.4 ± 0.7 µg GAE mg^{-1} and the *P. canaliculata* 101.2 ± 2.5 followed by *A. nodosum* (87.9 ± 2.0 µg GAE mg^{-1}) and F. *spiralis* (80.1 ± 1.3 µg GAE mg^{-1}) (Fig. 6.11). The lowest phenolic content in unfiltered ethanol extracts was found in *L. digitata* (3.5 ± 0.2 µg GAE mg^{-1}). Following filtration, highest levels of phenolic compounds were *F. vesiculosus* at 103.9 ± 1.5 µg GAE mg^{-1} and *P. canaliculata* at 65.85 ± 1.7 µg GAE mg^{-1} followed by *A. nodosum* (49.2 ± 0.2 µg GAE mg^{-1}) and *F. serratus* (43.4 ± 0.1). Again, the lowest level of phenolic compounds in filtered extracts was *L. digitata* (3.9 ± 0.1 µg GAE mg^{-1}) (Fig. 6.11)

In a similar way, the subfractions of the *F. vesiculosus* extract were examined for total phenolic content. The highest levels of phenolic contents in the unfiltered sub-

fractions of *F. vesiculosus* were in the tannin fraction with 515.7 ± 5.0 µg GAE mg^{-1} and the 3.5 - 100 kDa fraction with 419.7 ± 4.9 The lowest levels of phenolic compounds were found in the sugars' fraction with 16.9 ± 0.5 µg GAE mg^{-1}). The highest TPC levels in (0.45 µm) filtered sub-fractions were in the tannin fraction with 446.3 ± 9.9 µg GAE mg^{-1} and the 3.5-100 kDa fraction with 360.7 ± 4.73 followed by the 100 + kDa fraction (229.91 ± 5.55 µg GAE mg^{-1}) and the 0-3.5 kDa fraction (30.9 ± 0.3 µg GAE mg^{-1}). The lowest recorded level of total phenolic compounds in the filtered sub-fractions was seen in the sugar fraction with 16.91 ± 0.46 µg GAE mg^{-1}) (Fig. 6.12)

6.5 Discussion.

Despite improvements in the way our food is prepared, cooked and stored, the number of reported cases of food-related illnesses is on the rise. In addition, there has been a growing incidence of resistance to conventional antimicrobials that are used to treat food borne infections [27]. The contamination of RTE foods most often occurs during the post-processing stage of preparation. The thermal treatment of RTE food, that contains cooked meat, during processing is sufficient to eliminate *L. monocytogenes* but recontamination can occur during the handling, slicing and packaging phases of food production through cross-contamination [28]. RTE foods contain salts (sodium chloride, nitrite and nitrate) that have antimicrobial activity but these do not inhibit the growth of *L. monocytogenes* when food is refrigerated at 4 °C [29]. Chemical and natural preservatives, such as nisin and pediocin, are often used to inhibit *Listeria* in dairy and meat products, but *Listeria* can become highly resistant to these measures. For this reason, the development of new biopreservatives to protect against listeria and to prolong the shelf-life food is both desirable and necessary. Algae are known to produce a great multitude of biologically active secondary metabolites that have been shown to have anti-microbial activity. These bioactives have primarily been discovered in brown and red seaweeds and accordingly the screening of seaweed extracts for antimicrobial activity is a subject that has been frequently addressed in scientific literature. Here, ethanol extracts from ten brown seaweeds were screened for activity against a panel of both Gram-positive and Gram-negative pathogens using a microtitre plate-based dilution method. The pathogens *L. monocytogenes, S. aureus, E. coli* and *S. enterica* serovar Typhimurium were chosen because they are among the most prevalent foodborne organisms, that not only affect the quality of food but also cause severe illness if contaminated food is ingested [2]. An *in vitro* antibacterial assay that is simple, reproducible, rapid, sensitive, and cost effective was required for screening seaweed fractions. Often the amount of purified compounds or fractions which are available for antimicrobial screening can be a limiting factor in any workable screening programme [30]. In this assay, bacterial cells were grown to early log phase (OD_{600nm} of 0.3) as actively growing cells are more sensitive to antimicrobials than stationary cells from an overnight culture. The growing cells and different seaweed extracts were incubated together for two hours before the addition

of any media, as the components of growth media such as NaCl, carbohydrates etc., have been shown to inhibit the activity of certain antimicrobial peptides. The addition of double strength BHI broth to each experimental well was to facilitate the growth of any surviving bacteria in the presence of the extract.

The initial screening demonstrated that several of the ethanol extracts demonstrated moderate activity against the Gram-positive bacteria *S. aureus,* against the Gram-negative bacteria *E. coli,* and against *S. enterica* serovar Typhimurium at 24 h. An extended lag phase was observed for *S. aureus* (*F. serratus, F. vesiculosus, F. spiralis,* and *L. digitata), S. enterica* serovar Typhimurium (*F. vesiculosus, Fucus serratus, L. hyperborea,* and *F. spiralis),* E. coli O157:H7 (*F. spiralis* and *L. digitata)* and *E. coli 6235* (*F. vesiculosus).* A definite inhibition of growth was evident, however, each strain was able to overcome the inhibitory effect in time. Several extracts did, however, demonstrate potent inhibitory activity against the Gram-positive pathogen *L. monocytogenes*. According to the Food Safety Authority of Ireland (FSAI) *L. monocytogenes* poses a most challenging threat to the safety of food products in Ireland [25] making it a pertinent target of antimicrobial screening studies. The ethanol extracts from the seaweeds *A. nodosum, F. vesiculosus, F. serratus, F. spiralis, H. elongata and P. canaliculata* all significantly inhibited ($p<0.05$) the growth of *L. monocytogenes* 5788 measured after 24 h. The percentage inhibition for the three *Fucus* species as well as for *A. nodosum* was more than 90% with the most potent activity observed with the *F. vesiculosus* extract. No viable colony forming units (CFUs) were recovered at any dilution for these extracts indicating a complete inhibition of the *L. monocytogenes*. Based on the results of this initial screen, the ethanol 80% extract of *F. vesiculosus* was selected for further evaluation of its antimicrobial activity against *Listeria monocytogenes*. As different strains of the same bacterial species can vary greatly in their sensitivity to a given antimicrobial substance [5] the crude *F. vesiculosus* (Fv) extract was tested against three further strains of *Listeria* to access the specificity of the anti-listerial activity. At a working concentration of 1 mg/ml, percentage inhibition against all additional strains (*L. monocytogenes* 4590, *L. monocytogenes* 4608 and *L. monocytogenes* EGDe) exceeded 90% demonstrating that the crude Fv extract has potent antimicrobial activity against a range of *L. monocytogenes* strains and activity was not limited to a particular strain. This is an important characteristic for any putative antimicrobial agent to be used for

food preservation as a biopreservatives. It was also shown that Fv extract retained its potent antimicrobial activity at a concentration of 100 µg/ml but only displayed weak inhibition at 10 µg/ml. The positive control used in this study for Gram-positive bacteria was Nisin from *Lactococcus lactis* (2.5% w/w). Interestingly, at a concentration of 100 µg/ml the percentage inhibition of *L. monocytogenes* 5788 growth by the crude extract after 24 h was significantly higher than that of the positive control. Plate counts performed after 24 h revealed that no viable CFU was recovered from Fv extract (100 µg/ml) plates, while a count of listerial cells on the nisin (100 µg/ml) plates was comparable to control numbers. This indicates that at an extract concentration of 100 µg/ml, the crude Fv extract had a greater inhibitory effect on listerial growth than the positive control. However, it must be clearly stated that the working concentration for the nisin control was only 2.5 µg/ml.

Size exclusion dialysis is a valuable technique with which compounds can be fractionated by molecular weight through selective diffusion of molecules across a semipermeable membrane. The resulting fractions can then be tested individually or in combination for observed biological activities found in the crude sample. Anti-listerial activity was concentrated in the low (0 - 3.5 kDa) and medium (3.5 - 100 kDa) molecular weight fractions. Both fractions were active against every strain of *L. monocytogenes* tested with the 3.5 - 100 kDa fraction being active in some cases below 10 µg/ml. Again, during the analysis of subfraction activity, the nisin control was only consistently active against strains of *L. monocytogenes* at 1 mg/ml and failed to significantly inhibit growth at concentrations less than 100 µg/ml. Significant anti-listerial activity was also observed for the Tannin fraction and the high (100 + kDa) molecular weight fractions. Highly potent anti-listerial activity was observed with the 100 + kDa fraction but owing to the excessive amounts of pigmentation within the extract, consistent results were difficult to obtain by readings of optical density. The only subfraction not to demonstrate antimicrobial activity was the sugar subfraction.

Higher levels of antimicrobial activity from seaweed extracts have been reported in literature against Gram-positive bacteria [25] in agreement with data obtained in this study for the Gram-positive bacterium *L. monocytogenes*. As such it was decided to further test the crude *F. vesiculosus* extract and the Fv 3.5 - 100 kDa subfraction against the Gram-positive *S. aureus.* At 1 mg/ml, the Fv 3.5 - 100 kDa completely inhibited the growth of one of the strains (*S. aureus* Sa45) while the other

strain (*S. aureus* FRI913) had its growth greatly attenuated for the first 1 h. The effect of the crude Fv extract on these strains was mixed. The growth *S. aureus* Sa45 was arrested by the Fv crude extract for the initial 10 h while *S. aureus* FRI913 had its log phase delayed by approximately 4 h. Without doubt, Fv extracts have an inhibitory effect on the growth of *S. aureus*. However, the effect seems to be varying between the different strains and not as potent as the effect observed with *Listeria monocytogenes*. Potent antimicrobial activity against *S. aureus* has also been reported by [31] using methanol extracts of *Haligra* spp. This extract possessed significantly higher concentrations of total phenolic content than other seaweeds in the study. Also, significant inhibition of growth ($p>0.05$) by the crude Fv ethanol extract at 1 mg/ml was also observed for the Gram-negative bacterium *E. coli* 6235. Although the *E. coli* cells were not completely inhibited, growth was arrested for several hours before the cells entered log phase. Gram-negative pathogens tend to be more resistant to antimicrobials, in general, than Gram-positives owing to their additional outer membrane. The activity of seaweed extracts against Gram-negative pathogenic bacteria warrants further investigation as the problem of increasing bacterial resistance to antimicrobials becomes more pressing.

The term phenolic compound describes several hundred molecules found in edible plants that have in their structure a benzene ring substituted by at least one hydroxyl group [32]. Seaweeds are an excellent source of polyphenols which play an important role in preventing the degradation of lipids by free radicals. Phlorotannins are a group of phenolic compounds that are composed entirely of polymers of phloroglucinol. They have been identified in several brown seaweed families such as *Fucaceae,* Sargassaceae and *Cystoseiraceae [33]*. It had been reported that they are the only phenolic group detected in brown seaweeds. However, the presence of other phenolic compounds such as catechins, flavonols and flavonol glycosides have been described in methanol extracts of red and brown algae [34]. Phlorotannins are derived from the oligomerising decoupling of the phloroglucinol monomer. They are essential for early cell-wall development in members of the *Fucaceae* family and for reducing oxidative damage that occurs from UV radiation and fluctuations in nutrient availability. Phlorotannins are difficult to characterise, isolate and purify because of their polymeric structure and similar polarity [26]. The total phenolic content of 80% ethanol extracts from 10 brown seaweeds was tested using the Folin-Ciocalteu

method. This method measures the total concentration of phenolic hydroxyl groups present in a sample. Phenolic compounds in the seaweed extract react with the Folin-Ciocalteu reagent to form a blue complex that can be detected and quantified by reading absorbance at 735 nm [35]. The highest levels of phenolic compounds in filtered seaweed extracts were found in the *F. vesiculosus* extract (103.9 ± 1.5 μg GAE mg^{-1}) followed by *P. canaliculata*, *A. nodosum*, *F. spiralis* and *F. serratus*. The level of phenolic compounds in seaweed is directly affected by the climate in which they grow and the amount of sunlight that they are subjected to. As such, the same seaweed species harvested in different countries at different times of the year can have differing concentrations of phenolic compounds. An extension of this is that the levels of phenols in seaweeds are also affected by their location along the shoreline. Those seaweeds that are located higher up on the shore are exposed more often to sunlight/UV radiation and suffer more from desiccation than those species found lower down the intertidal zone and that spend more of their time submerged in the water. This results in those species found lower down requiring reduced levels of phenols for protection [24], while those located in the upper regions of the shore contain comparatively increased concentrations of phenols. The highest concentrations of phenolic compounds were consistently observed in those extracts from seaweed species inhabiting the upper to mid region of the shoreline (*P. canaliculata, A. nodosum F. vesiculosus, F. spiralis* and *F. serratus*). Filtered extracts from seaweeds found in the lower or subtidal zone of the shoreline were found to contain very low levels of phenols (< 20 μg GAE mg^{-1}). High levels of phenolic compounds correlated strongly with the observed antimicrobial activity of the ethanol extracts against several strains of *Listeria monocytogenes.* All seaweed extracts that demonstrated significant inhibition of *Listeria* ($p<0.05$) at 24 h and, whose percentage inhibition of growth was < 90%, contained high levels of phenolic compounds. All fractions of *F. vesiculosus* which were investigated for antimicrobial activity were also found to be rich in phenolic compounds. The highest concentration of phenolic compounds among the *F. vesiculosus* subfractions was found in the tannin fraction. In contrast, seaweed extracts with low levels of phenol compounds had no significant impact on the growth of *Listeria monocytogenes*.

Phlorotannins have been reported to occur commonly in the 10 - 100 kDa size range. However, large amounts of low molecular weight phlorotannins are known to

be found in *F. vesiculosus,* and in other related species. It has been reported that *F. vesiculosus* contains an array of phlorotannins less than 1.2 kDa in size [26]. The higher molecular weight fractions of *F. vesiculosus* (3.5 - 100 kDa and 100 + kDa) were found to contain between 3 and 4 times the polyphenol concentration of the crude Fv extract. The low molecular weight fraction (0 - 3.5 kDa) exhibited levels of polyphenol comparable with the crude *F. vesiculosus* ethanol extract. This is consistent with observed levels of activity. Polyphenols in brown algae are thought to provide a low level defense against bacteria. As high-molecular weight phlorotannins appear to be, at best only weakly antibacterial and antifungal, intermediate- and low-molecular weight fractions are presumably responsibly for these bioactivities [36]. From the results obtained here, we conclude that the antimicrobial activity of the crude *F. vesiculosus* extract and *F. vesiculosus* subfraction against *L. monocytogenes* is because of the presence of large quantities of low molecular weight polyphenolic compounds. This is agreement with several *in vitro* studies that have demonstrated that algal derived polyphenols and flavonoids exhibit antimicrobial activity [37]. Phenolic compounds can also act as antioxidants by chelating metal ions preventing radical formation and improving the endogenous antioxidant system [38]. Numerous studies have described a correlation between phenolic content, the inhibition of α-amylase and α-glucosidase, and antioxidant activity. For example, a study carried out by Lordan et al., [39] demonstrated that total phenolic content of extracts from *F. vesiculosus* and *Pelvetia canaliculata* correlated with their antioxidant activity.

6.6 Conclusion.

In conclusion, the results of this study indicate that the Irish seaweeds, *F. vesiculosus, F. serratus, F. spiralis, A. nodosum, P. canaliculata* and *H. elongata* successfully display antimicrobial activity which is correlated to the presence of large concentration of phenolic compounds. *F. vesiculosus* had the highest levels of phenolic compounds of all the seaweeds investigated and demonstrated the most potent antimicrobial activity against the pathogen *L. monocytogenes.* This is a promising outcome as to the potential of utilising such extracts from seaweeds in food products to serve as both biopreservation agents and antioxidants.

6.7 References.

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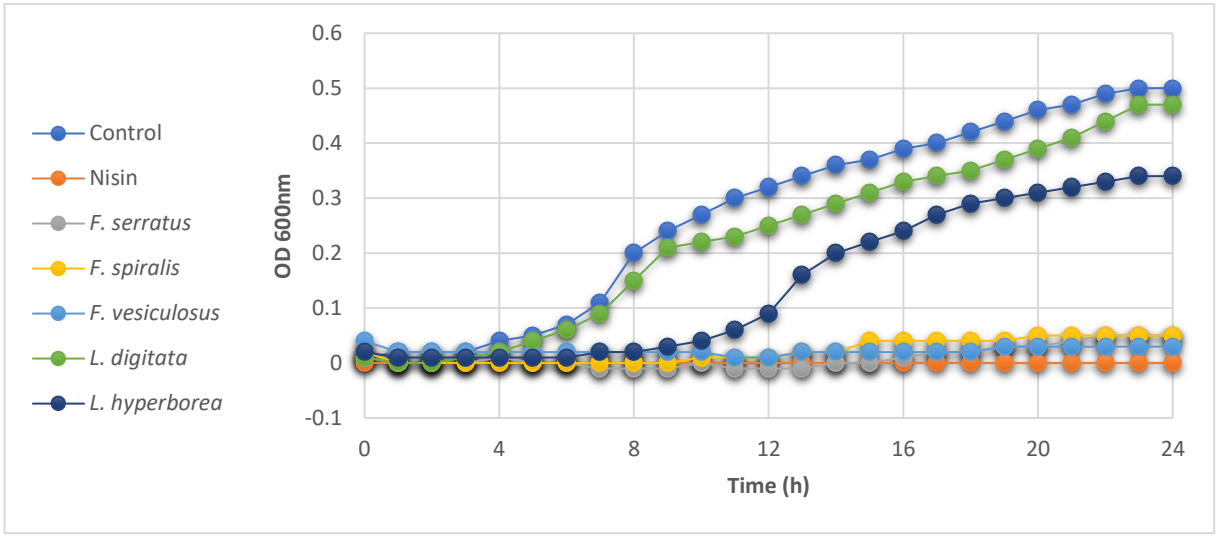
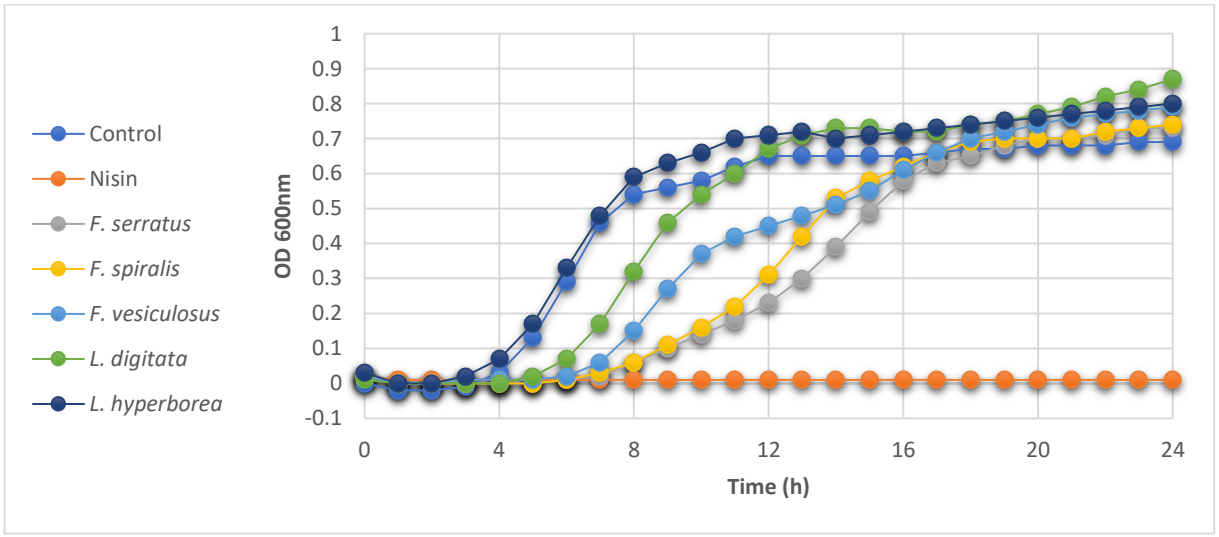
Figure 6.1. Antimicrobial activity of ethanol extracts of *Fucus serratus*, *F. spiralis*, *F. vesiculosus*, *Laminaria digitata* and *L. hyperborea* against (a) *Listeria monocytegenes* 5788, (b) *Staphylococcus aureus* 5236, (c) *Salmonella enterica* serovar Typhimurium 6236, (d) *Escherichia coli* O157H7, and (e) *E. coli* 6235 at a concentration of 1mg/ml. The control for the experiment was 2.5% Nisin from Lactococcus lactis at a concentration of 1 mg/ml.

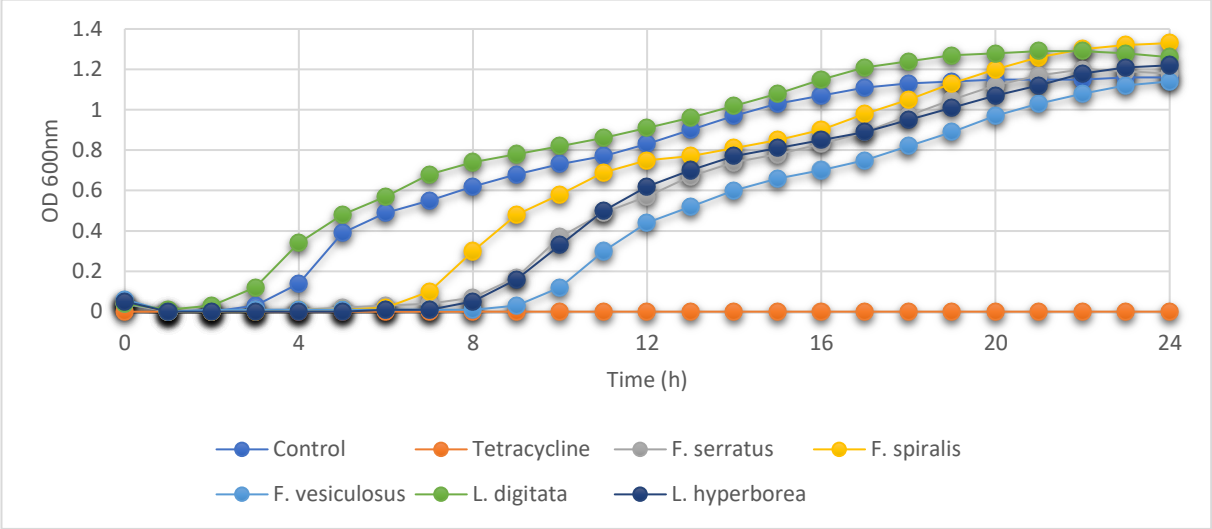
(a) *L. monocytogenes* 5788



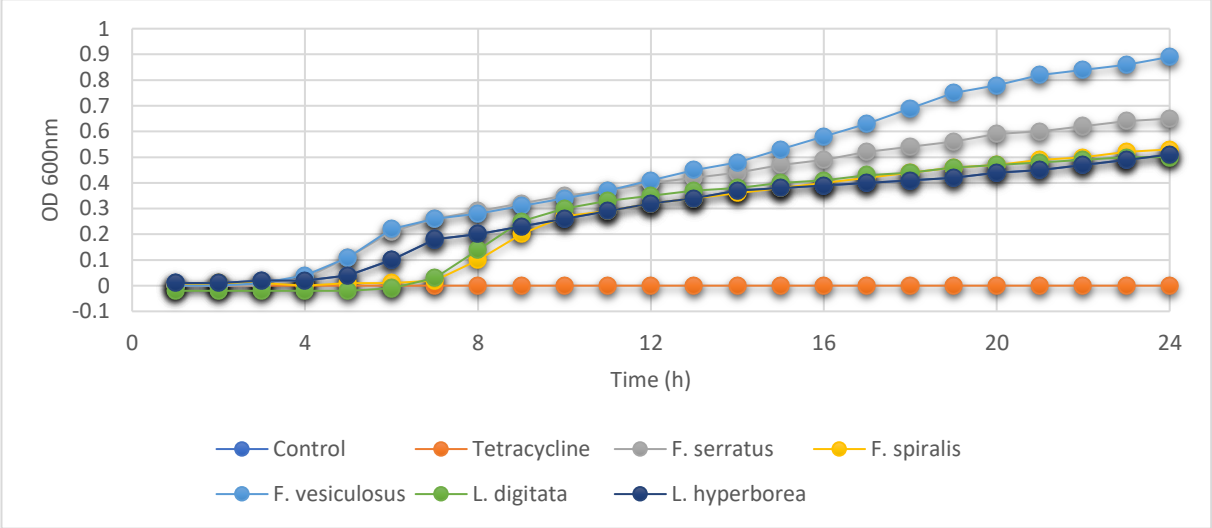
(b) *S. aureus 5236*



(c) *S. enterica* serovar Typhimurium 6236



(d) *E. coli O157H7*



(e) *E. coli 6235*

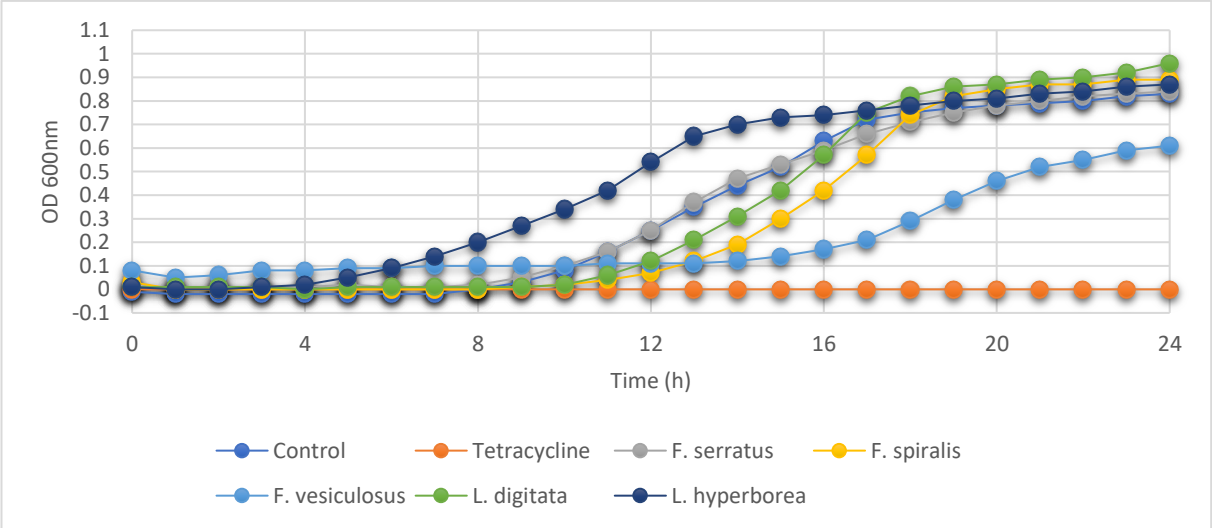
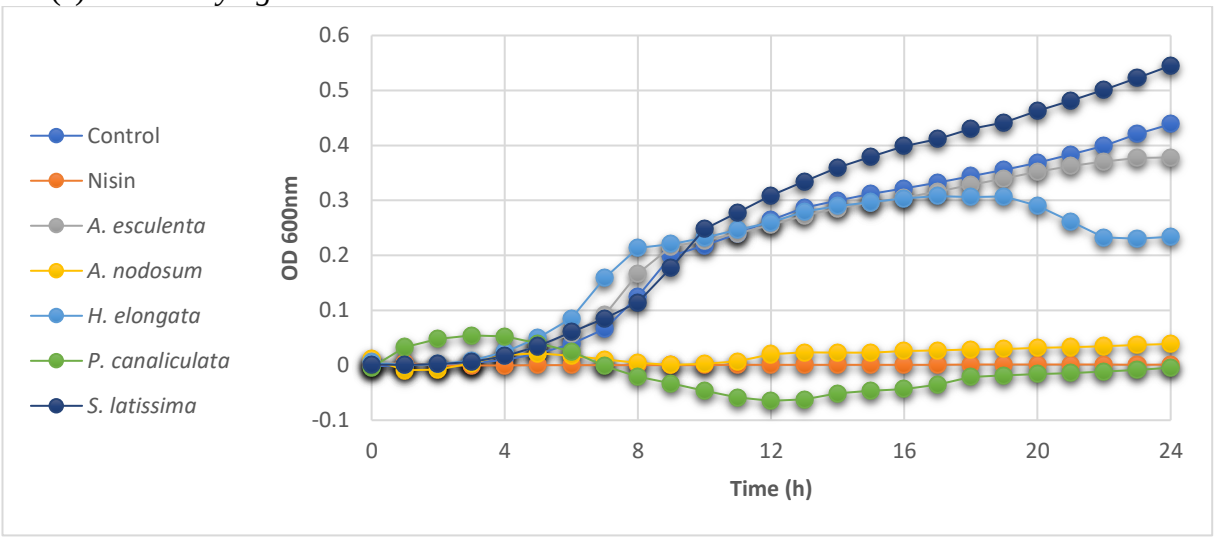
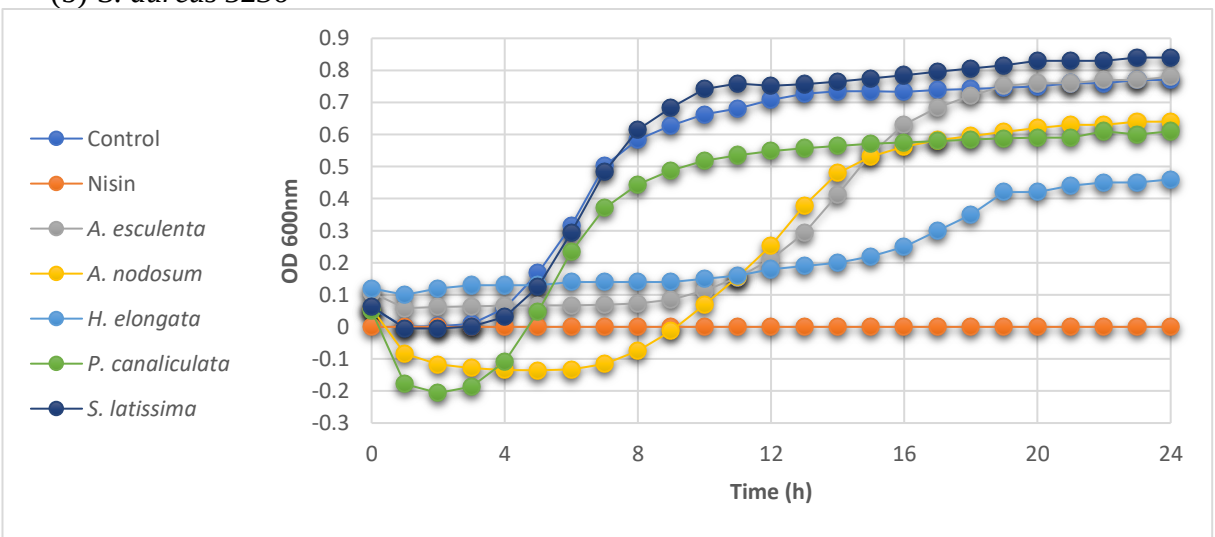


Figure 6.2. Antimicrobial activity of EtOH extracts of *A. esculenta*, *A. nodosum*, *H. elongata*, *P. canaliculata*, and *S latissima* against (a) *Listeria monocytogenes 5788*, (b) *Staphylococcus aureus 5236*, (c) *Salmonella enterica serovar Typhimurium 6236*, (d) *Escherichia coli O157H7*, and (e) *E. coli 6235*. at a concentration of 1mg/ml. The control for the experiment was 2.5% Nisin from *Lactococcus lactis* (2.5%) at a concentration of 1 mg/ml.

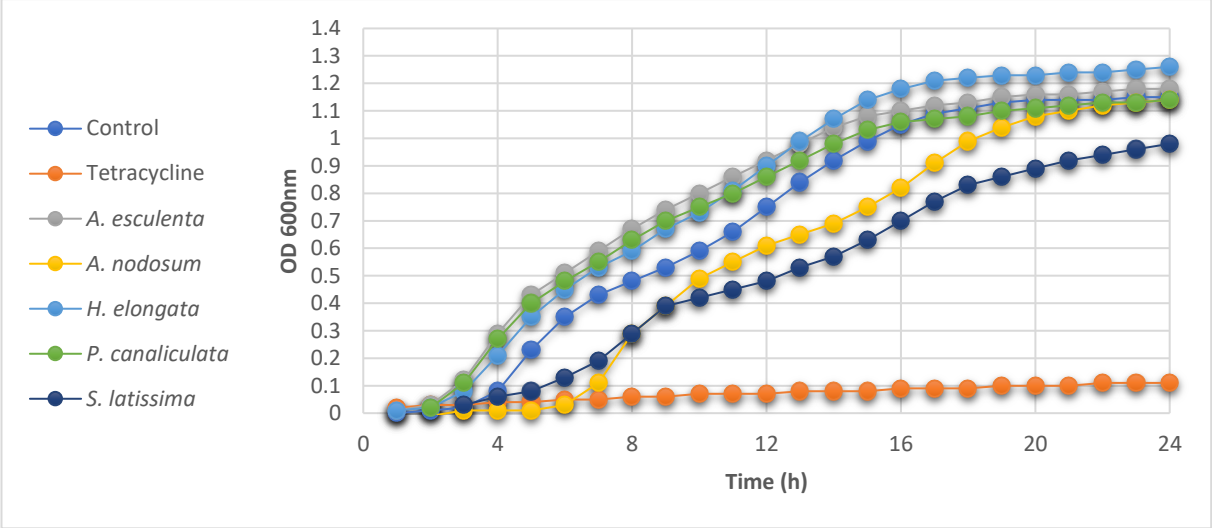
(a) *L. monocytogenes 5788*



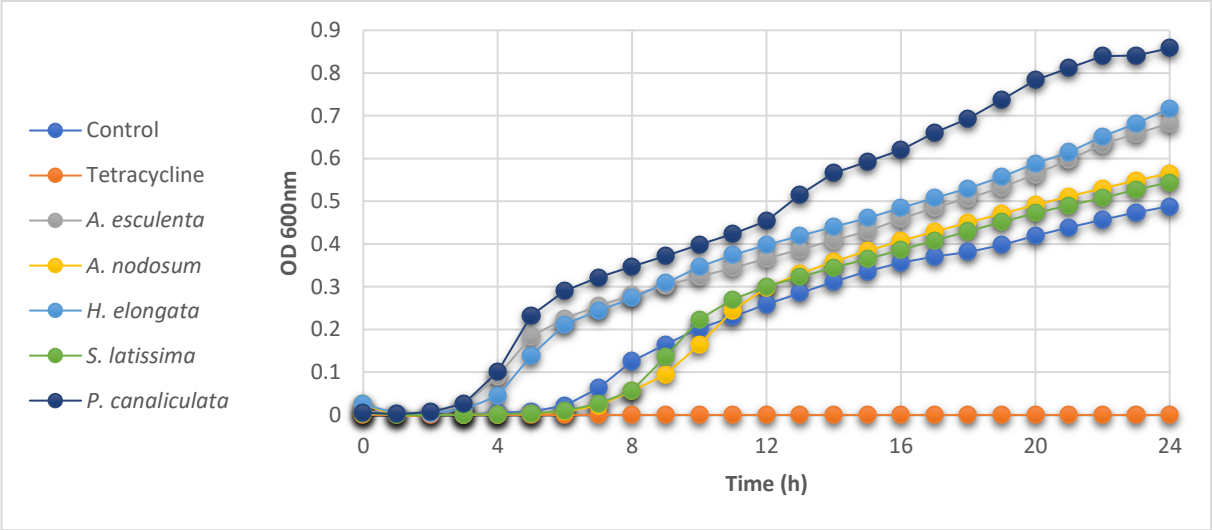
(b) *S. aureus 5236*



(c) *S. enterica* serovar Typhimurium 6236



(d) *E. coli O157H7*



(e) *E. coli 6235*

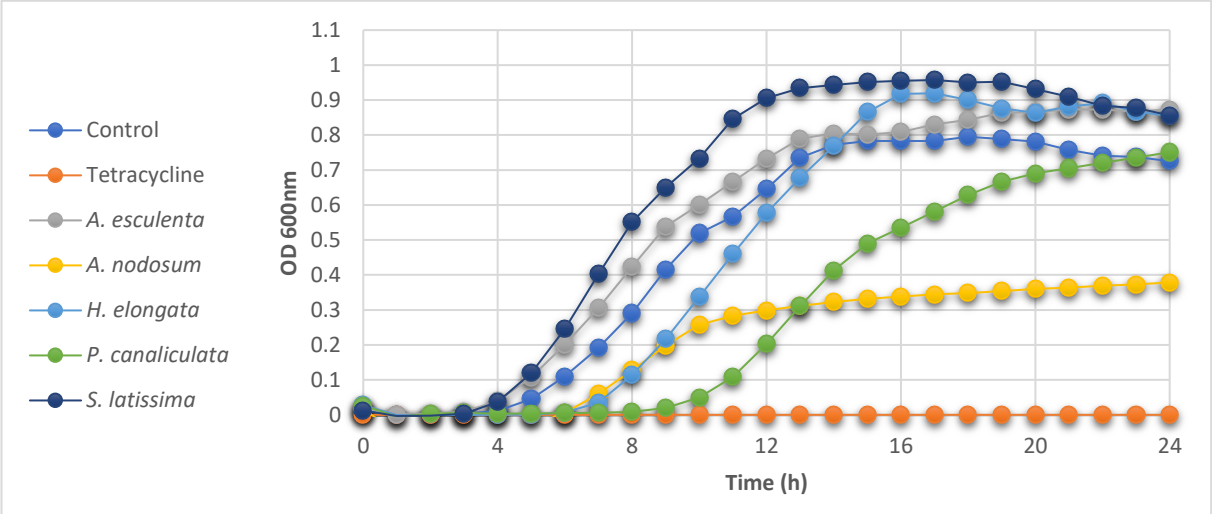
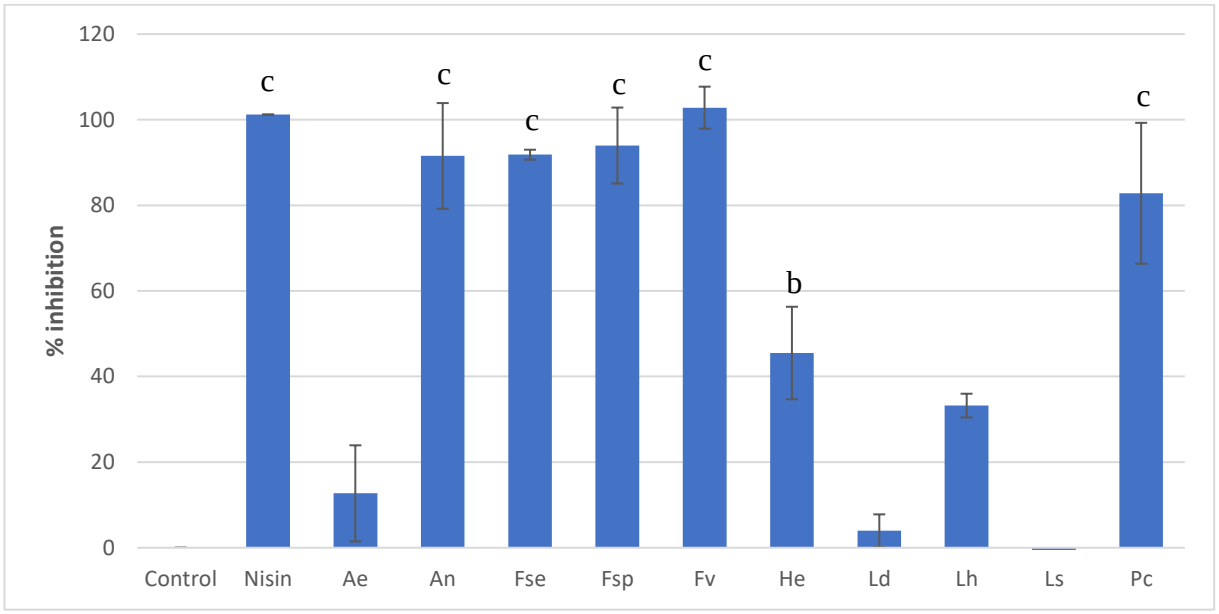
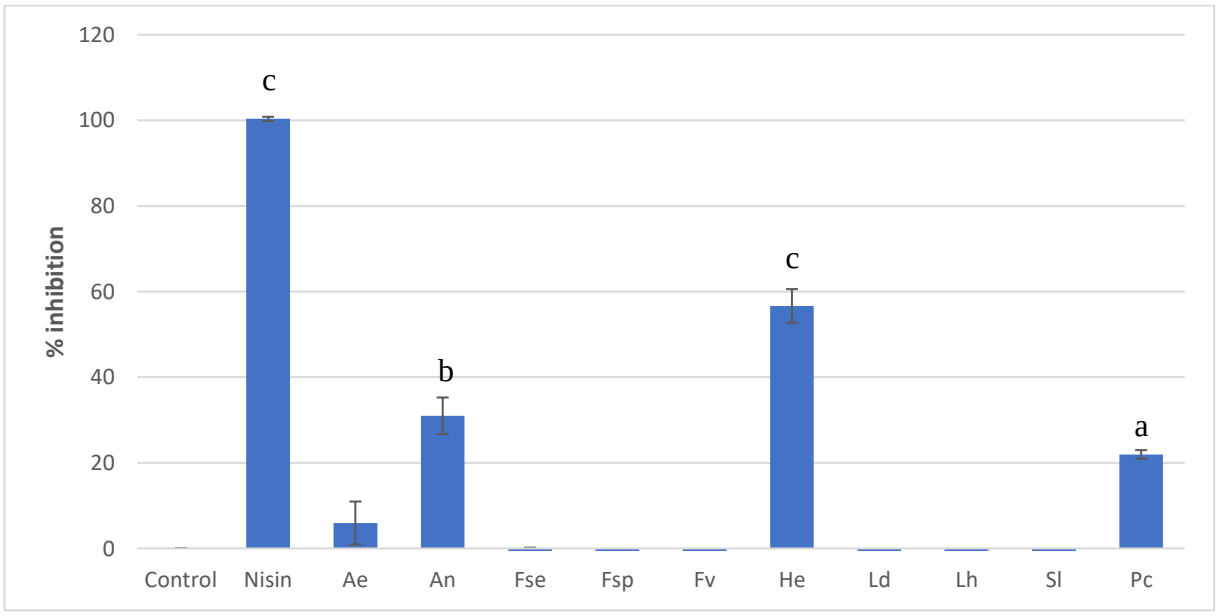


Figure 6.3 Percentage inhibition at 24 h of (a) *Listeria monocytogenes* 5788, (b) *Staphylococcus aureus* 5236, (c) *Salmonella enterica* serovar Typhimurium 6236, (d) *Escherichia coli* O157H7 and (e) *E. coli* 6235 in the presence of ethanol extracts derived from Irish brown seaweeds at a concentration of 1 mg/ml. Data represent the mean (±SE). (a = P<0.05), b = P<0.005, c = P<0.0005 relative to the control, un-paired T-test).

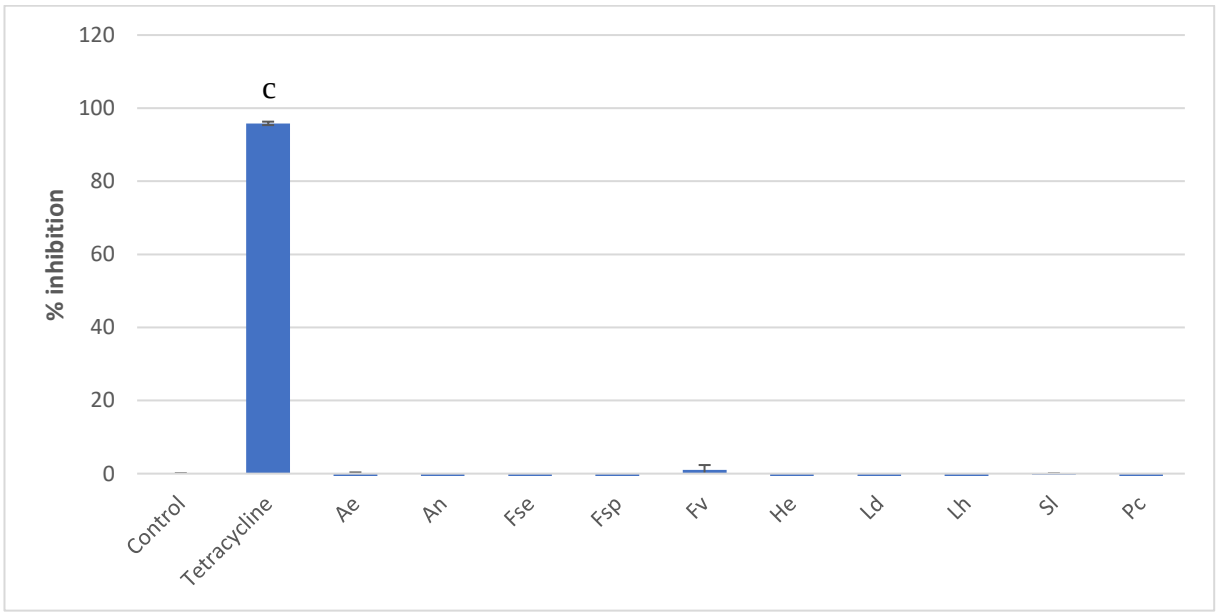
(a) *L. monocytogenes* 5788



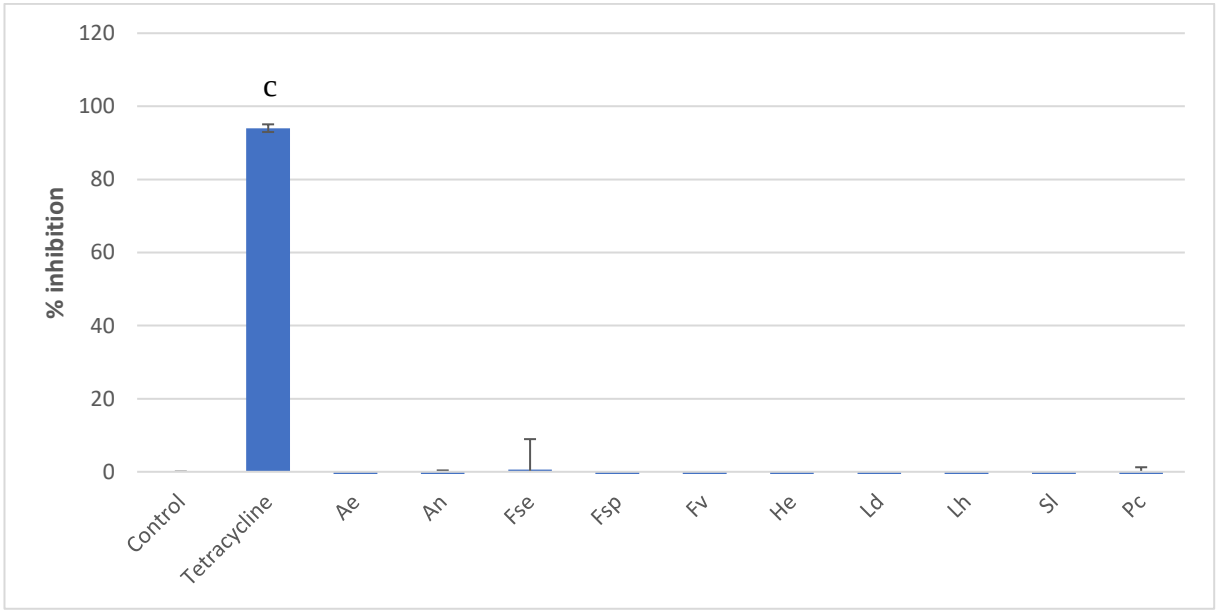
(b) *S. aureus* 5236



(c) *S. enterica* serovar Typhimurium 6236



(d) *E. coli* O157H7



(e) *E. coli* 6235

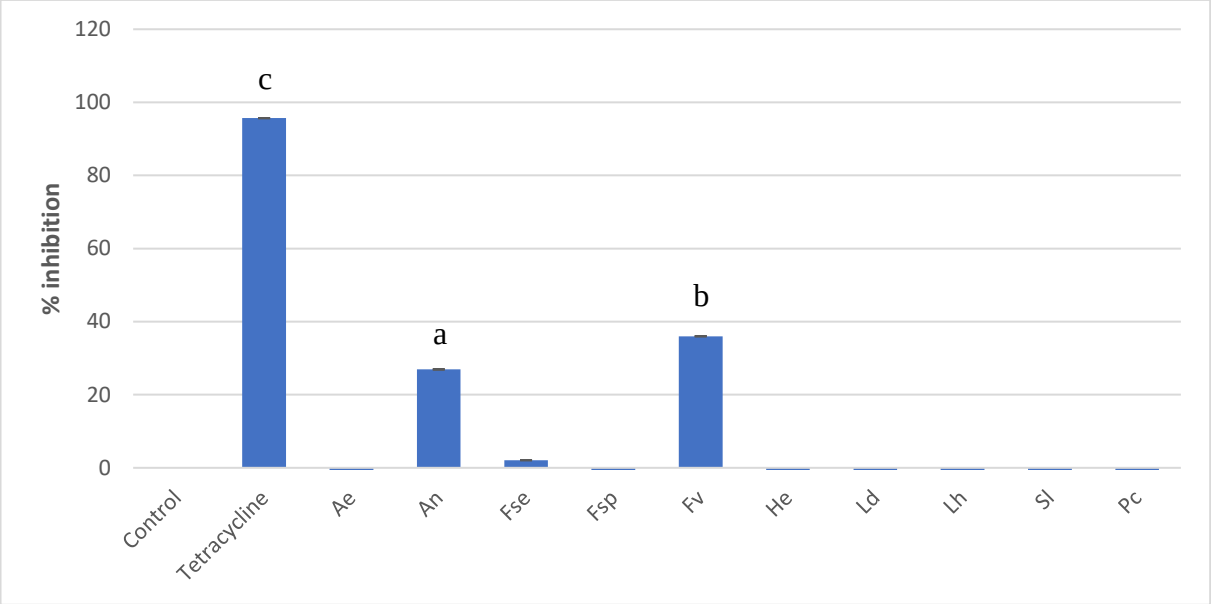
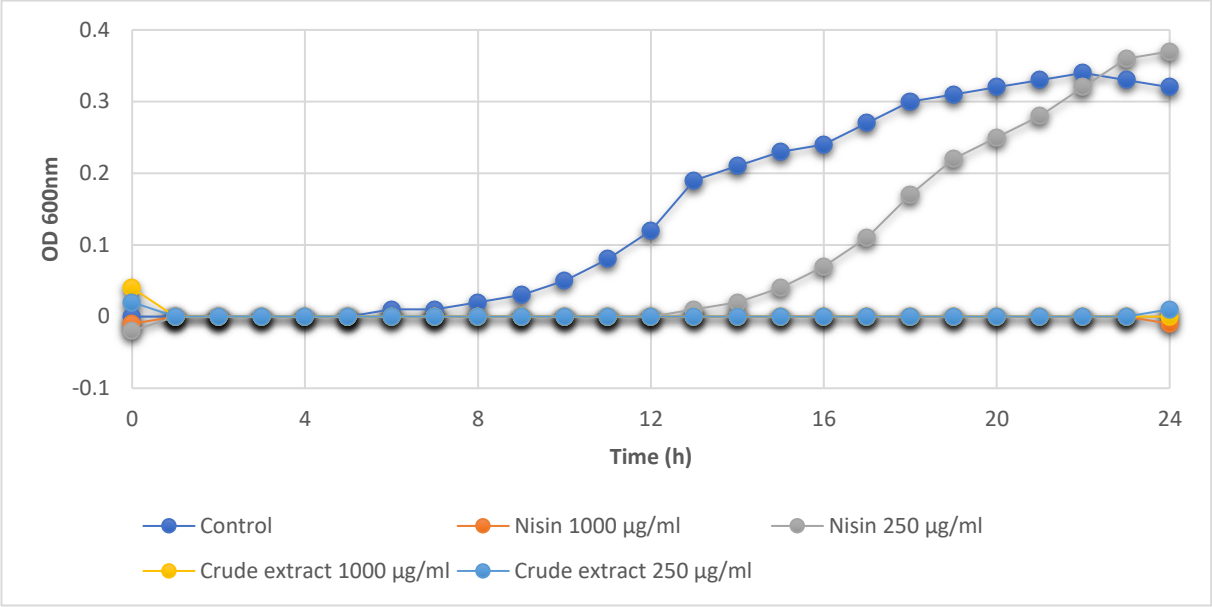
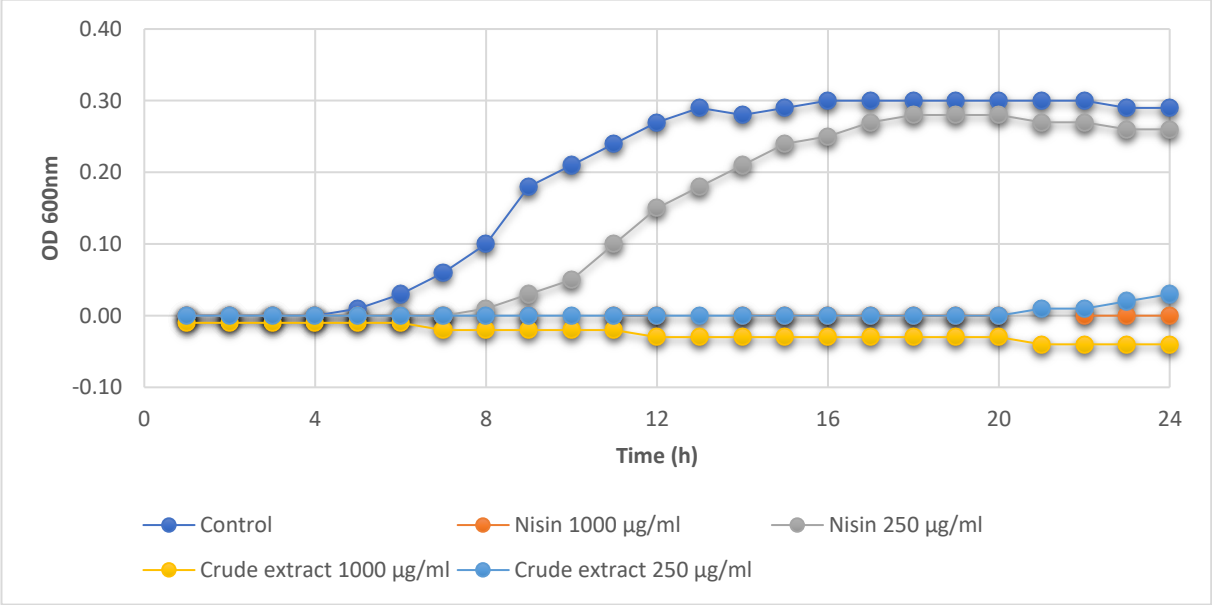

120
100
80
60
40
20
0
% inhibition
c
a
b
Control
Tetracycline
Ae
An
Fse
Fsp
Fv
He
Ld
Lh
Sl
Pc

Figure 6.4. Strain specificity of the anti-listerial activity of the crude *F. vesiculosus* extract at 1000 µg/ml and 250 µg/ml against (a) *L. monocytogenes* 4590 (b) *L. monocytogenes* 4608 and (c) *L. monocytogenes* EGDe. The control for the experiment Nisin (2.5%) from *Lactococcus lactis*

(a)



(b)



(c)

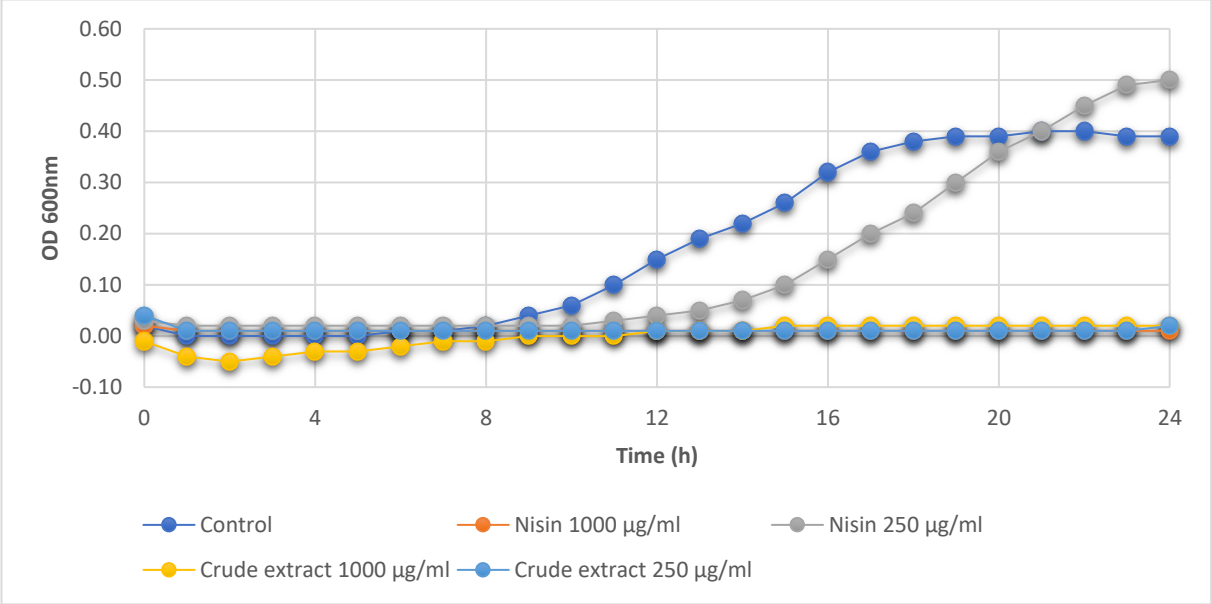
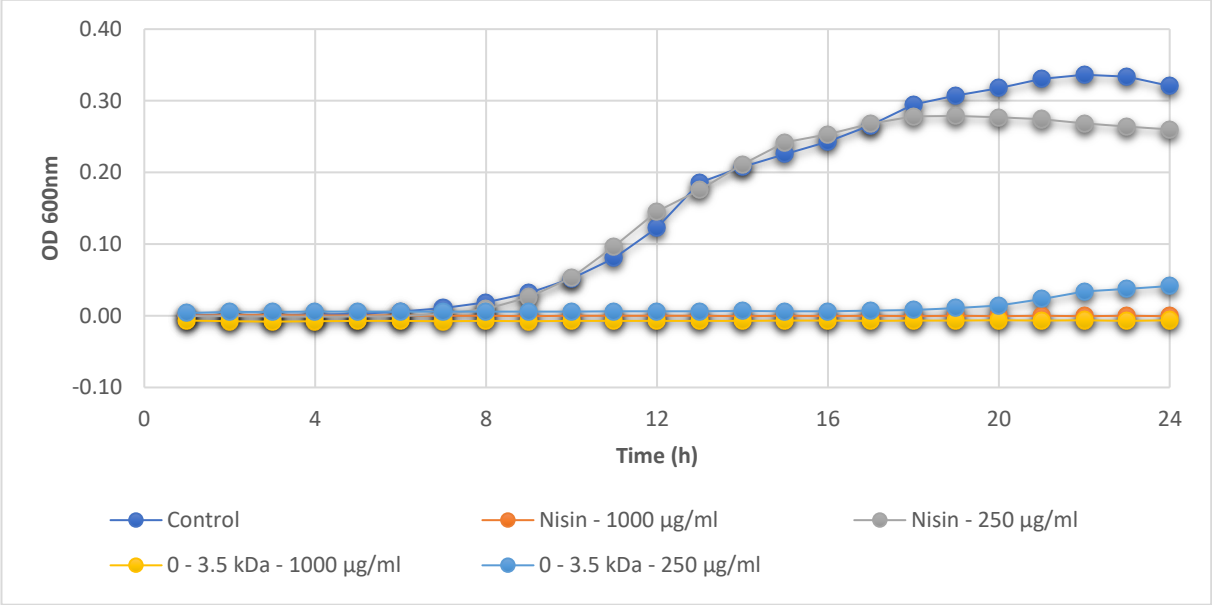
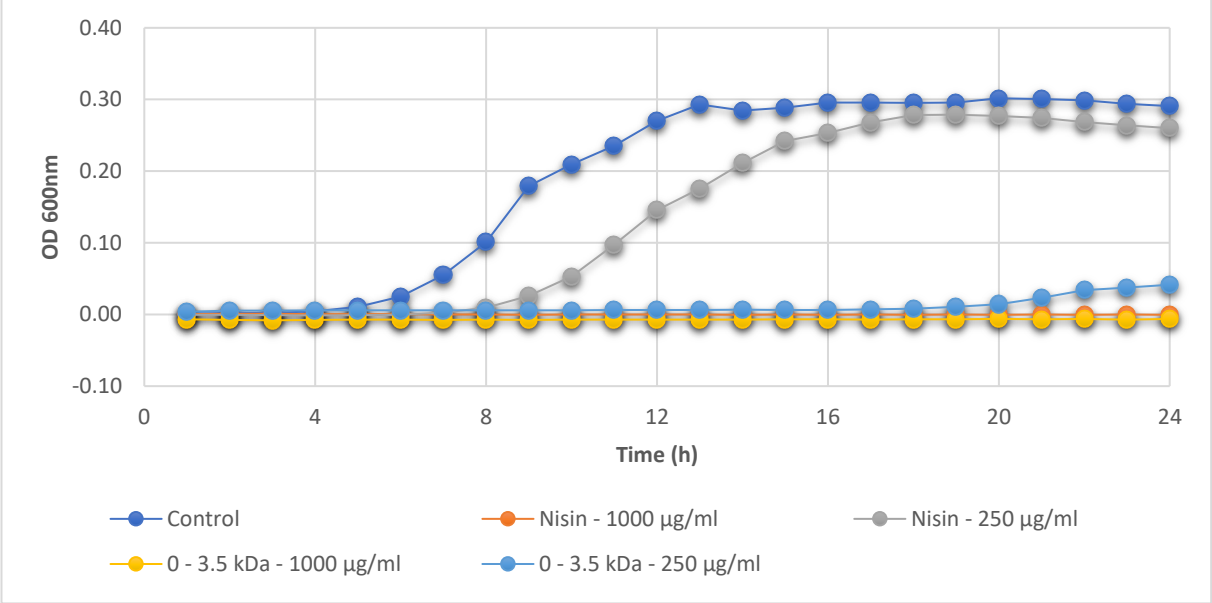

0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
OD 600nm
0
4
8
12
16
20
24
Time (h)
Control
Nisin 1000 µg/ml
Nisin 250 µg/ml
Crude extract 1000 µg/ml
Crude extract 250 µg/ml

Figure 6.5 Strain specificity of the anti-listerial activity of the crude *F. vesiculosus* 0 – 3.5 kDa fraction at 1000 µg/ml and 250 µg/ml against (a) *L. monocytogenes* 4590 (b) *L. monocytogenes* 4608 and (c) *L. monocytogenes* EGDe. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*.

(a) *L. monocytogenes* 4590



(b) *L. monocytogenes* 4608



(c) *L. monocytogenes* EGDe

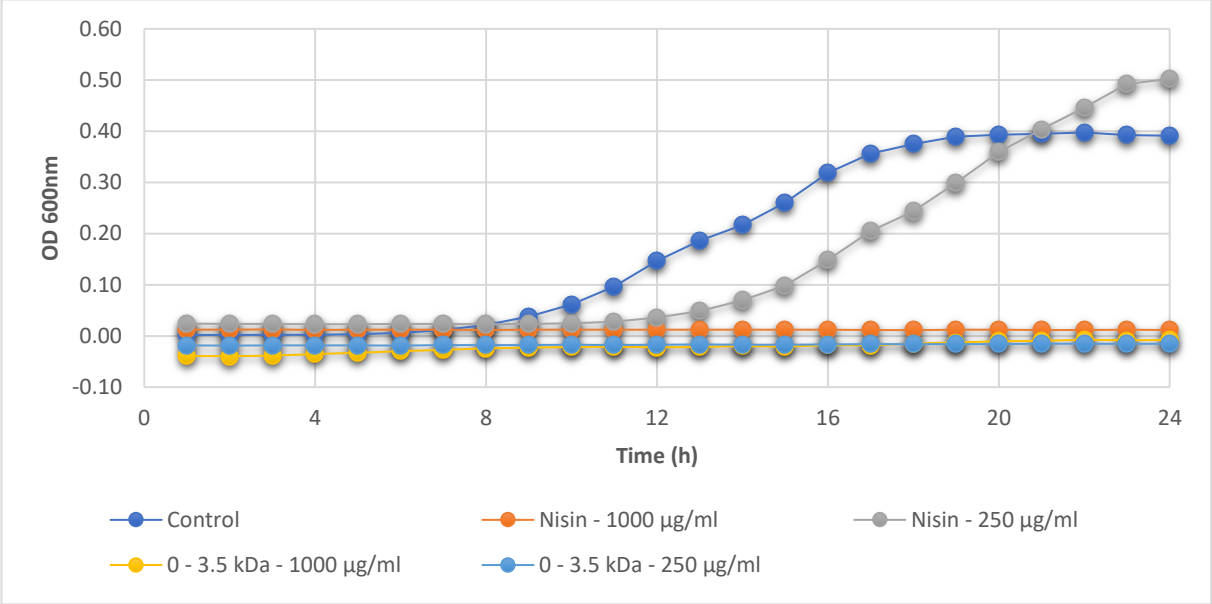
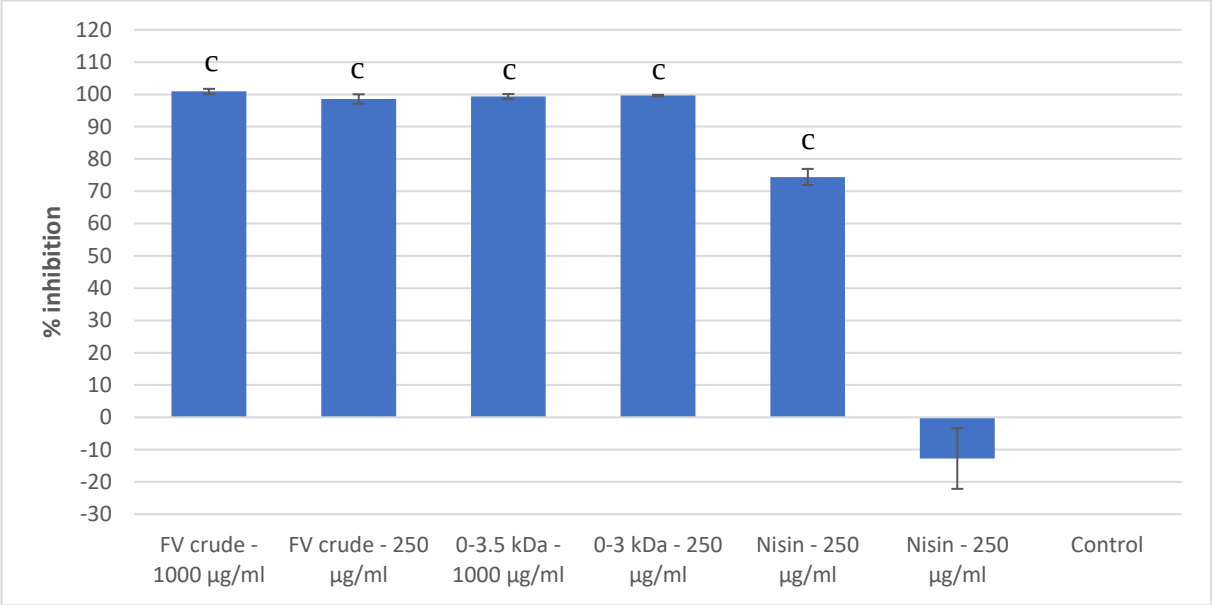
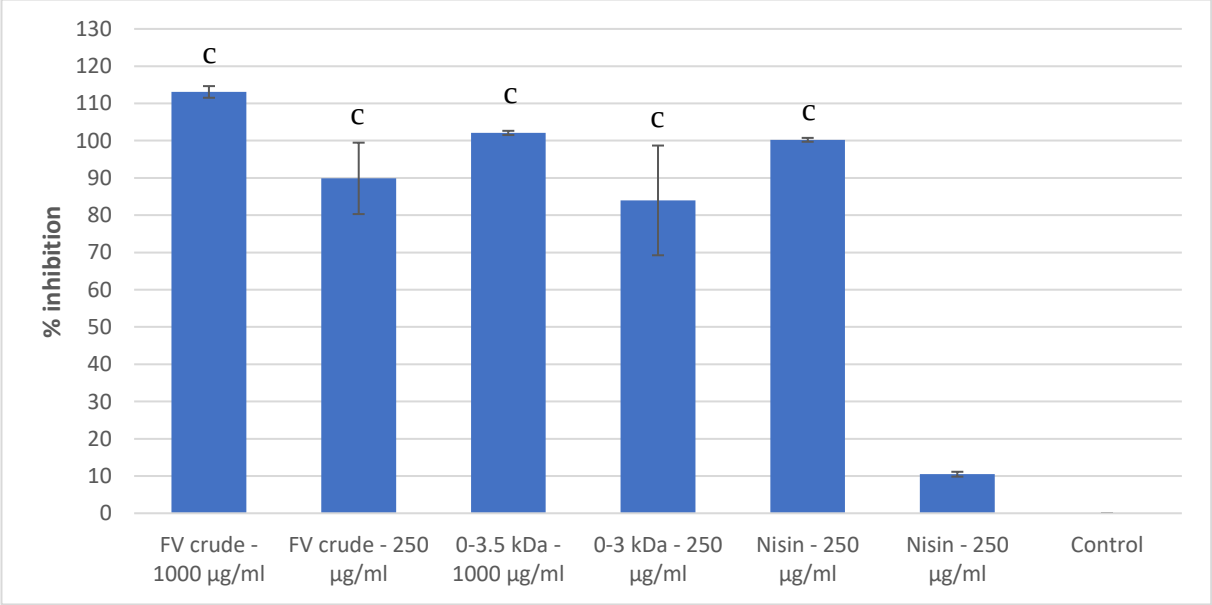


Figure 6.6 Percentage inhibition at 24 h (a) *L. monocytogenes* 4590 (b) *L. monocytogenes* 4608 and (c) *L. monocytogenes* EGDe, by the *F. vesiculosus* crude ethanol extract and the 0 – 3.5 kDa fraction. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*. (a = P<0.05), b = P<0.005, c = P<0.0005 relative to negative control.

(a) L. monocytogenes 4590



(b) *L. monocytogenes* 4608



(c) *L. monocytogenes* EGDe

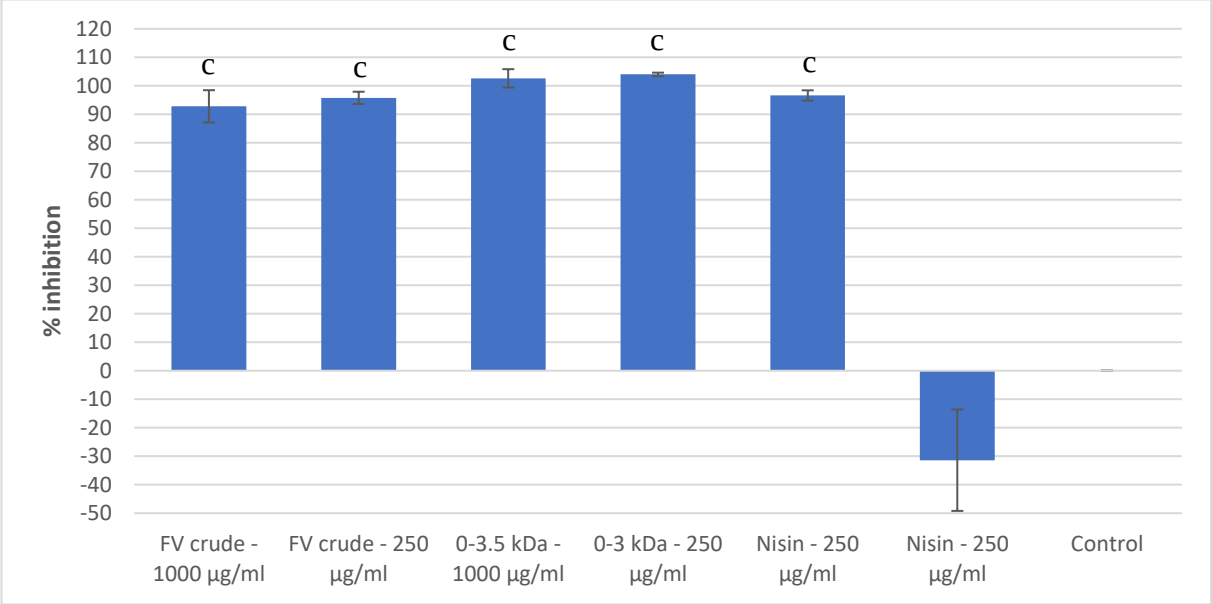
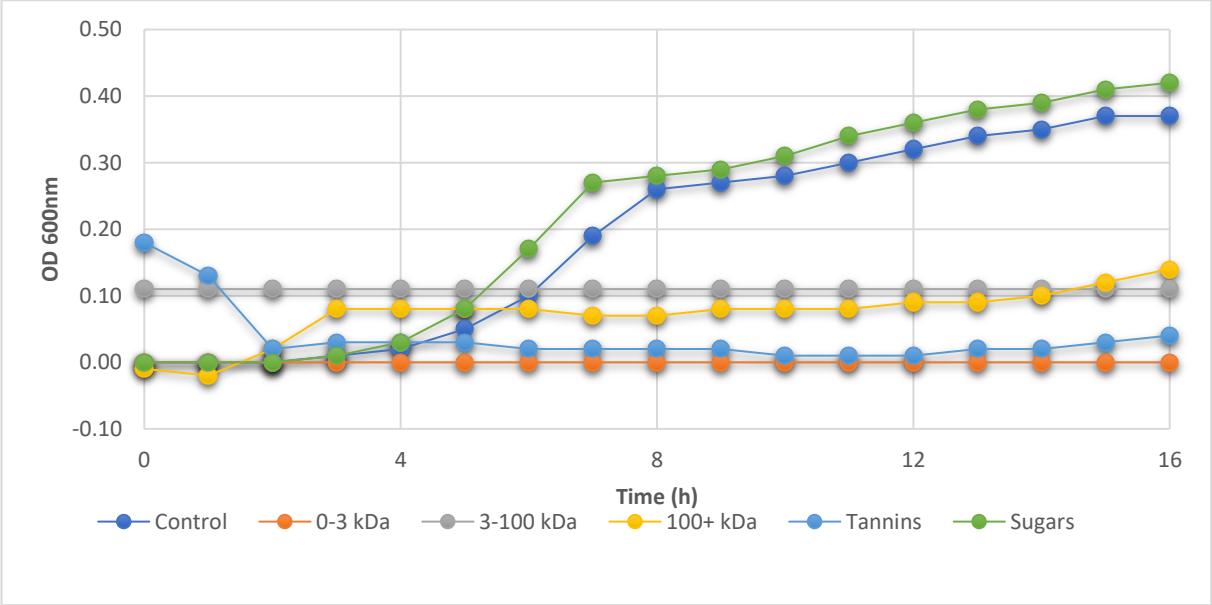


Figure 6.7 Different fractions of *F. vesiculosus* were tested for activity against (a) *L. monocytogenes* 5788 and (b) *L. monocytogenes* EDGe. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*. The working concentration of extract was 1 mg/ml.

(a) *L. monocytogenes* 5788



(b) *L. monocytogenes* EDGe

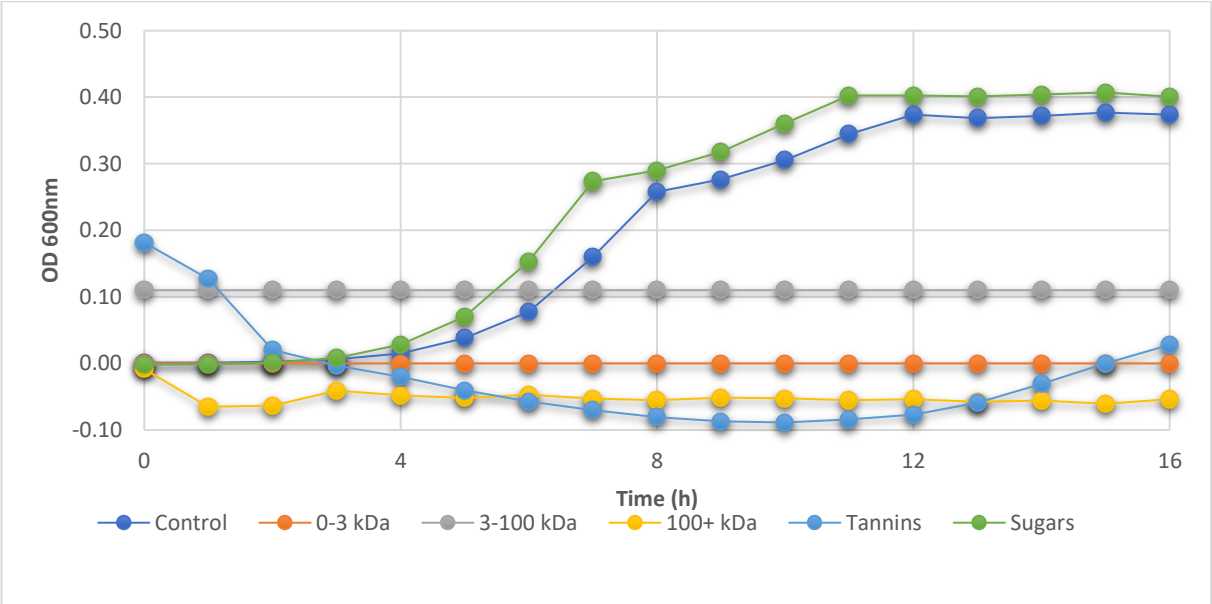


Figure 6.8 The *F. vesiculosus* fractions 0 – 3.5 kDa and 3.5 – 100 kDa were tested against a representative *L. monocytogenes* strain (5788) at 1000, 100 and 10 µg/ml. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*.

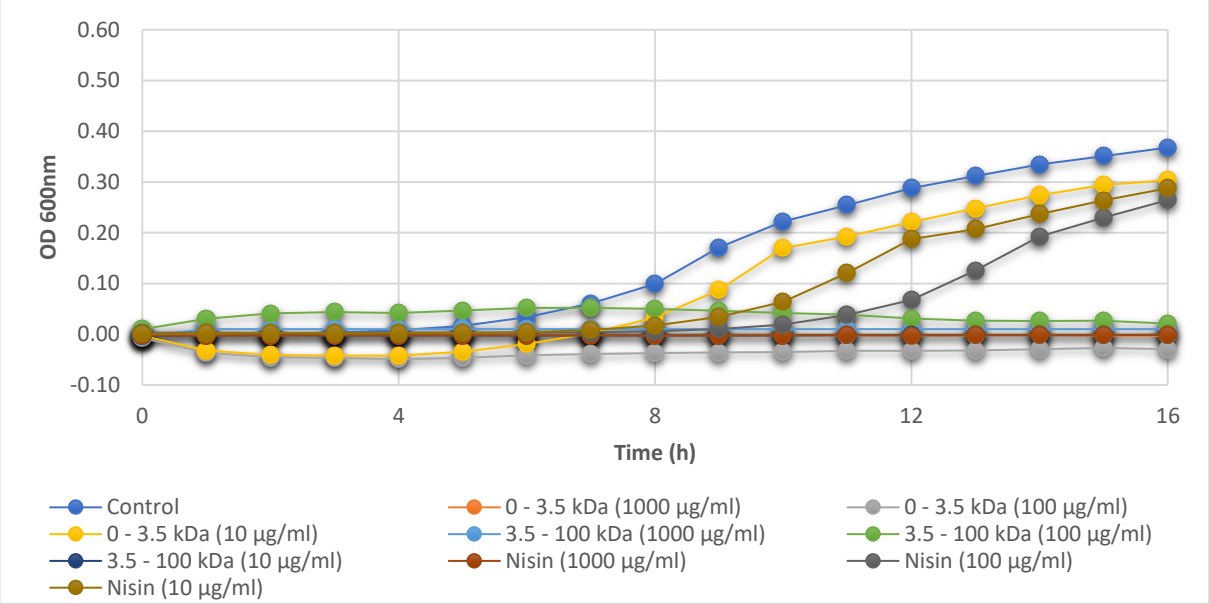
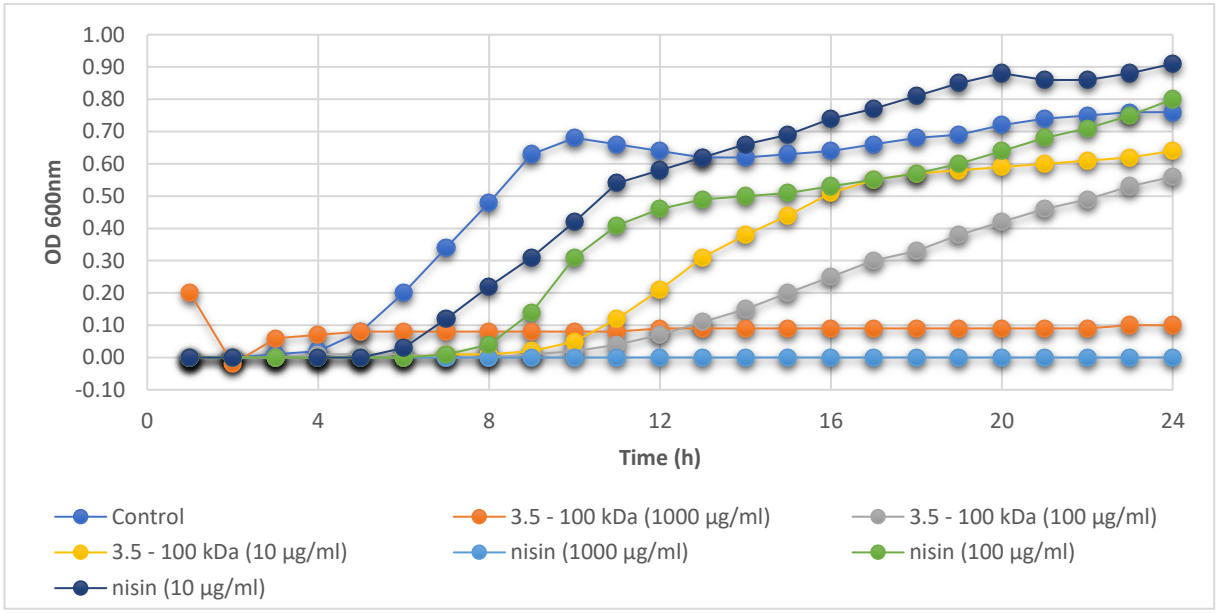
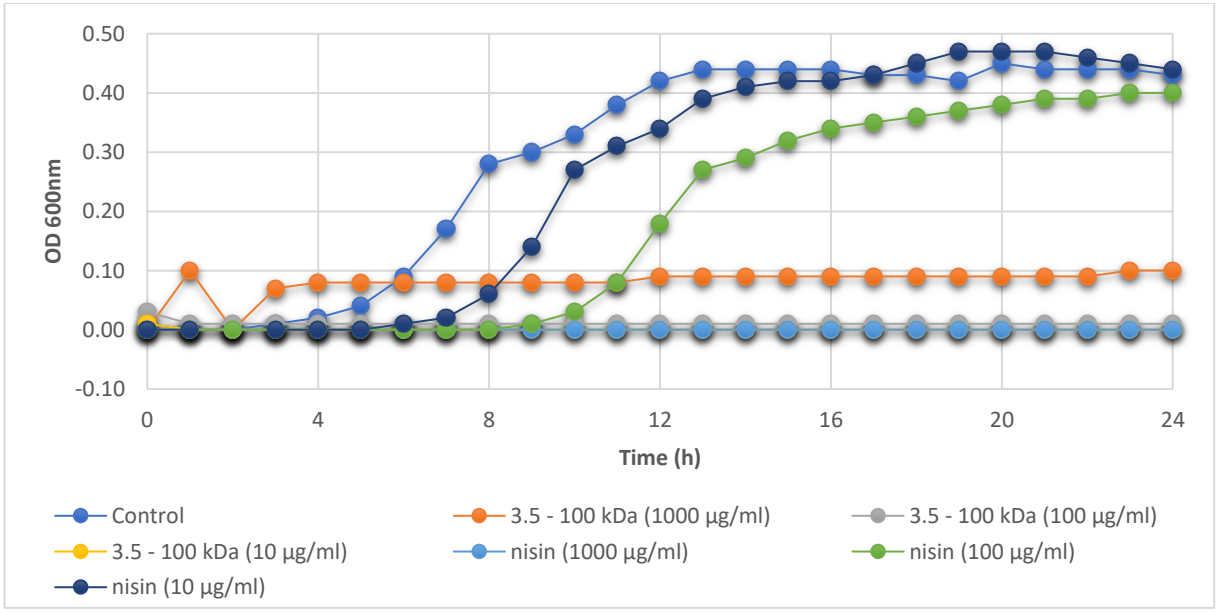


Figure 6.9 The effect of the *F. vesiculosus* 3.5 - 100 kDa subfraction had on the viability of (a) *L. monocytogenes* 5788, (b) *L. monocytogenes* 4590, (c) *L. monocytogenes* 4608, and (d) *L. monocytogenes* EGDe at concentrations of 1000, 100 and 10 µg/ml. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*.

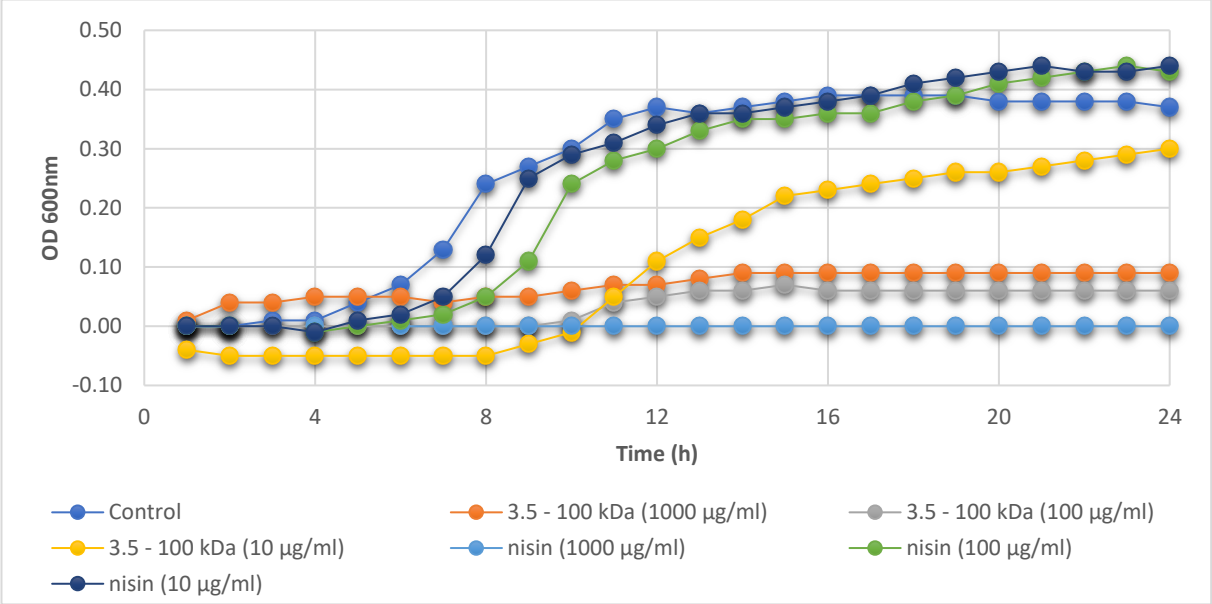
(a) *L. monocytogenes* 5788



(b) *L.monocytogenes* 4590



(c) *L. monocytogenes* 4608



(d) *L. monocytogenes* EGDe

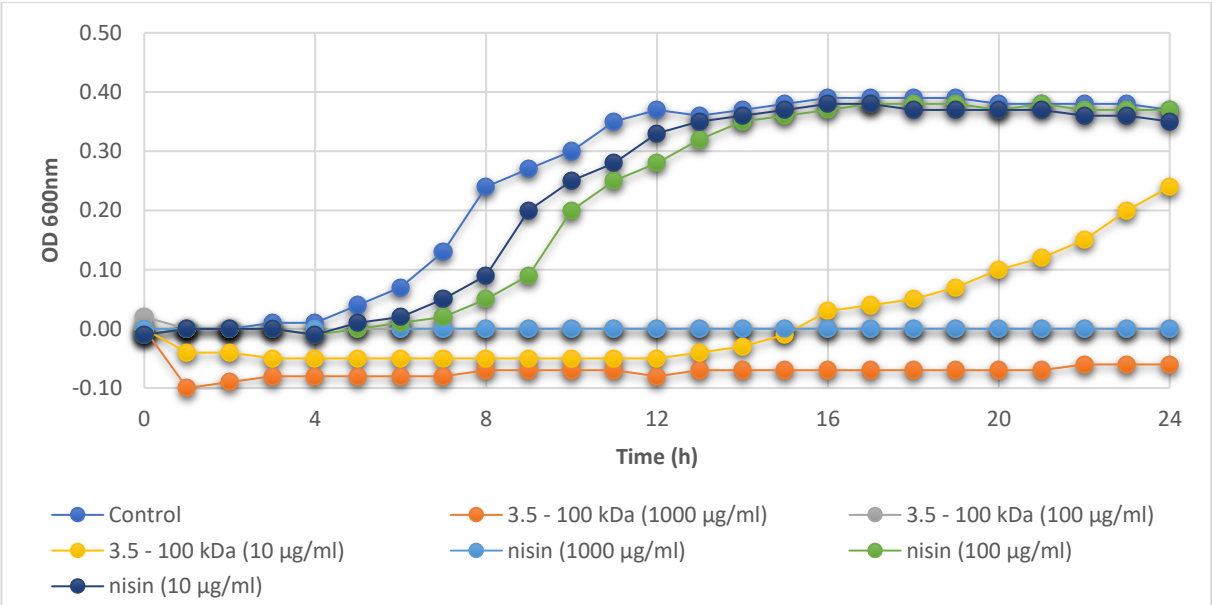
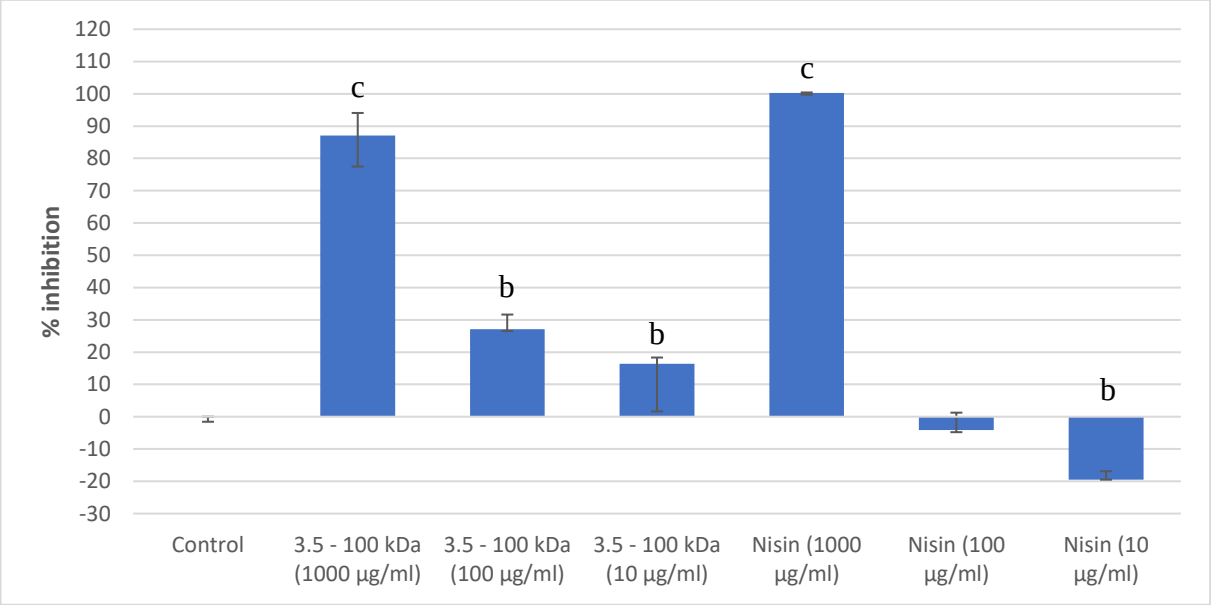
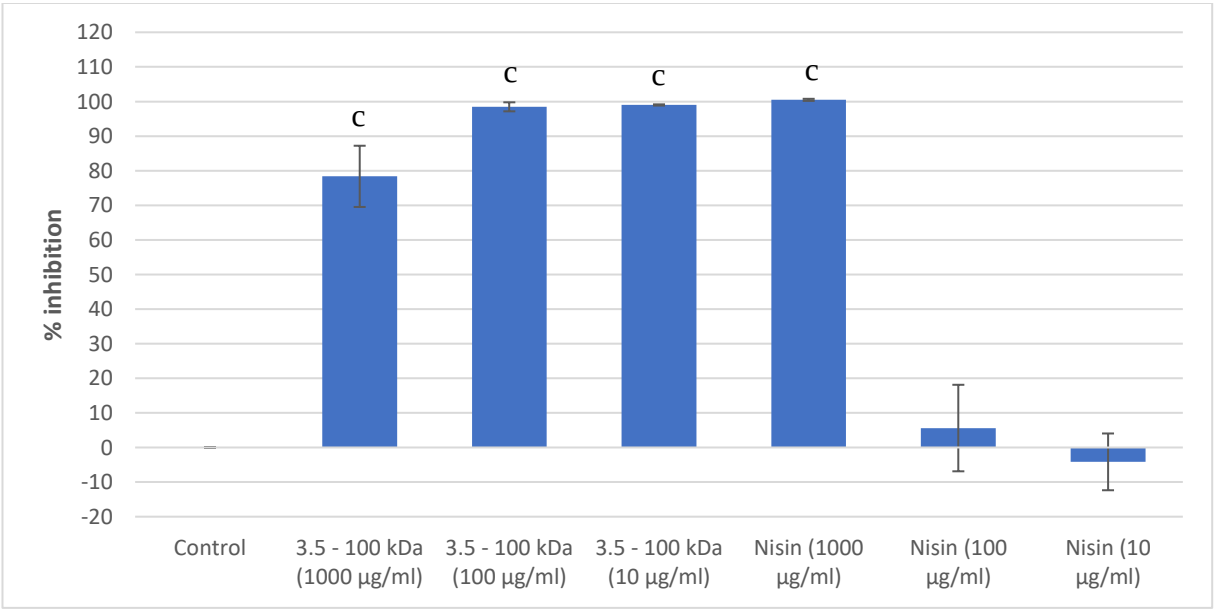


Figure 6.10. Percentage inhibition at 24 h by the *F.* vesiculosus 3.5 – 100 kDa fraction (a) *L. monocytogenes* 5788, (b) *L. monocytogenes* 4590 (c) *L. monocytogenes* 4608 and (d) *L. monocytogenes* EGDe. The positive control for the experiment was Nisin (2.5%) from *Lactococcus lactis*. (a = P<0.05), b = P<0.005, c = P<0.0005 relative to negative control.

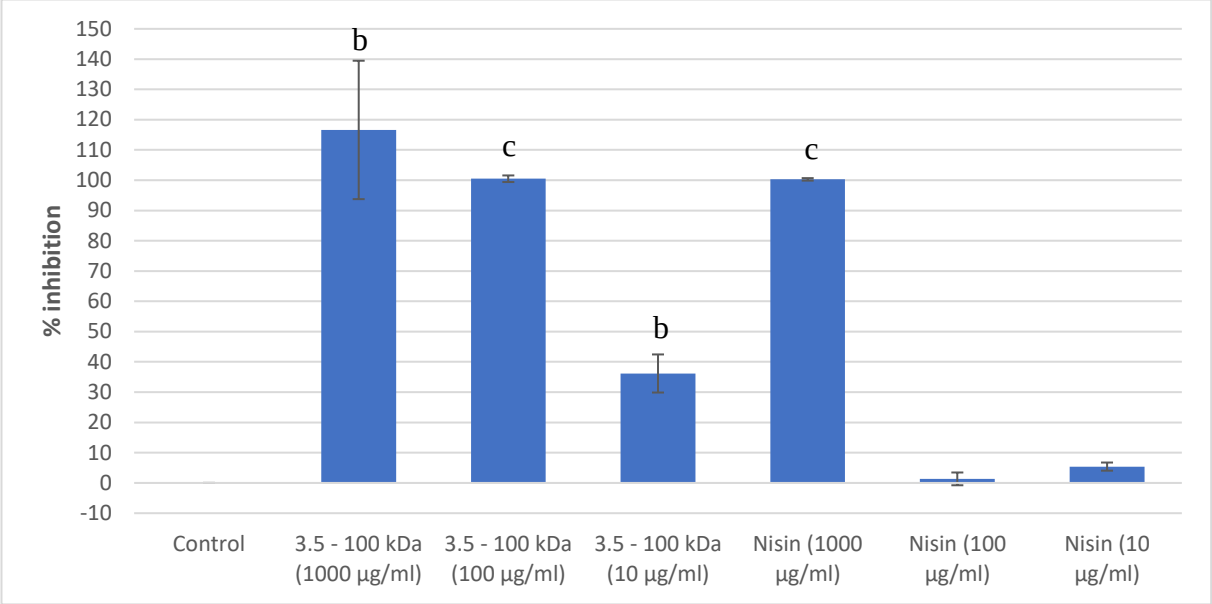
(a) *L. monocytogenes* 5788



(b) *L. monocytogenes* 4590



(c) *L. monocytogenes* 4608



(d) *L. monocytogenes* EDGe

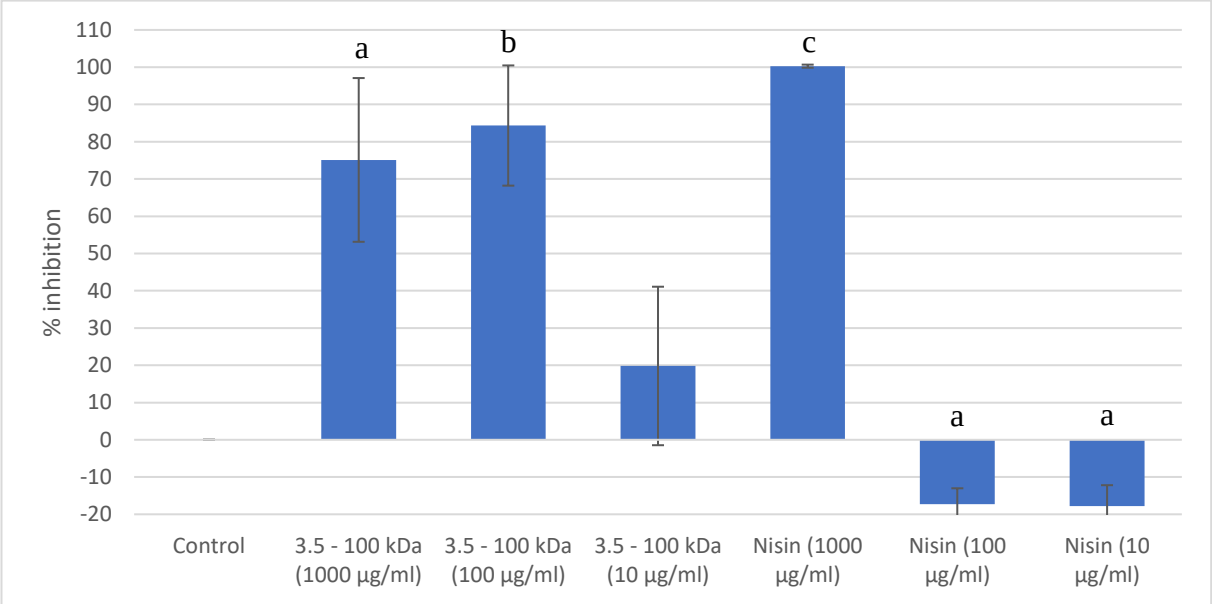


Figure 6.11. Total phenolic content of (a) unfiltered and filtered ethanol extracts from ten brown seaweeds. Values represent the mean (± SE).

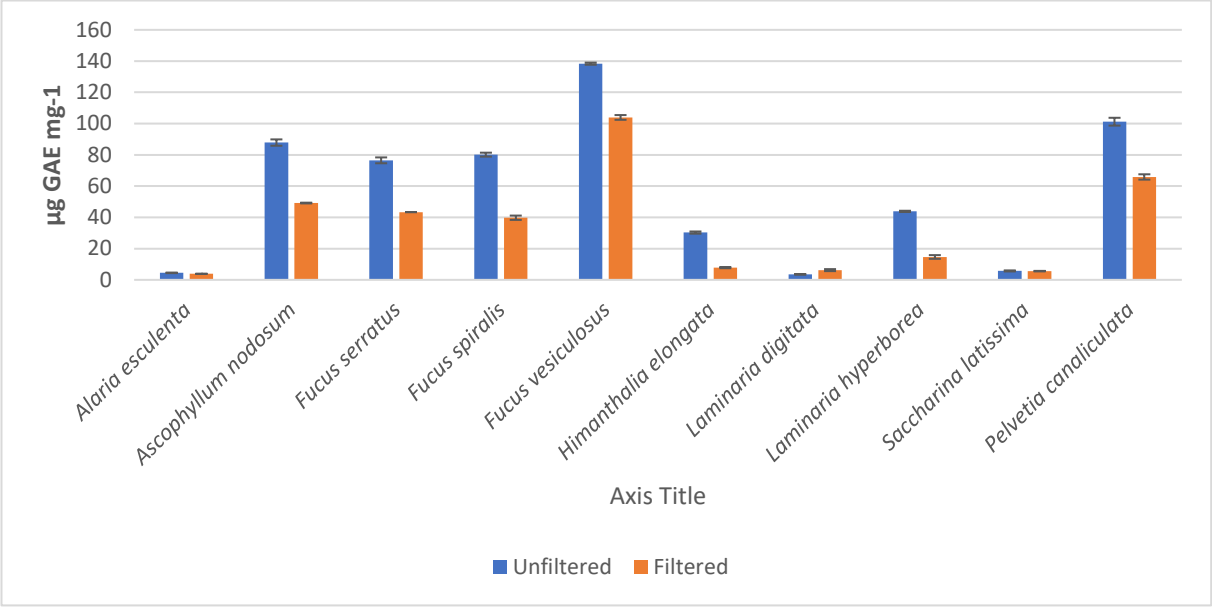
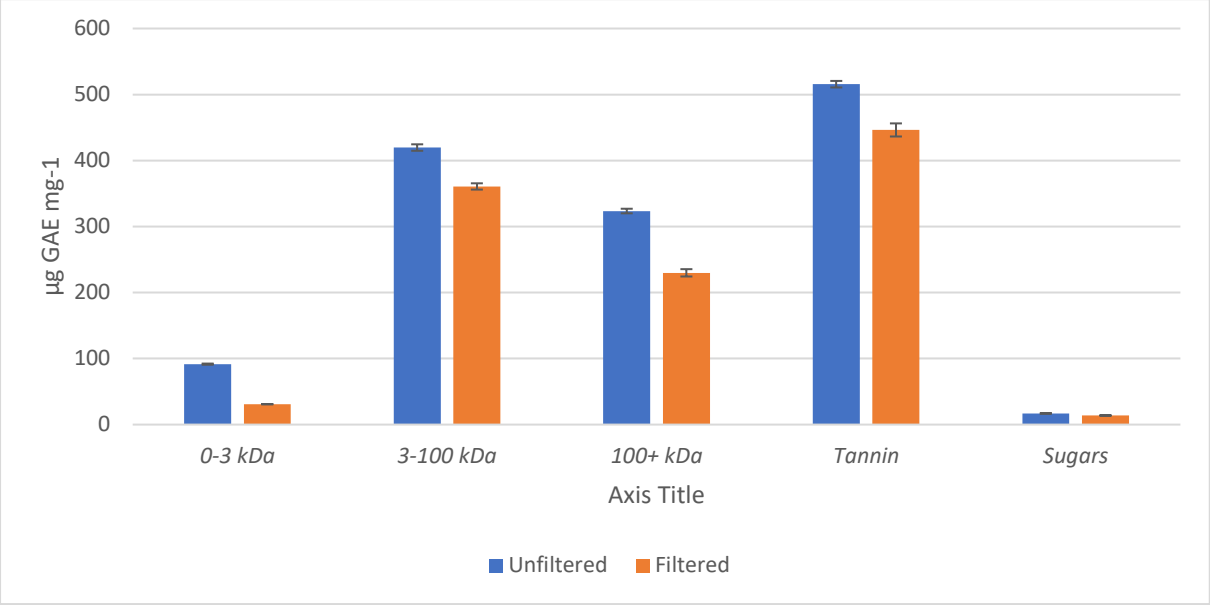


Figure 6.12 Total phenolic content of unfiltered and filtered subfractions of *F. vesiculosus.* Values represent the mean (± SE).



7.1 General Discussion.

Seaweeds are multicellular marine algae that belong to three distinct evolutionary groups: brown seaweeds (phylum Ochrophyta, class Phaeophyceae), red seaweeds (phylum Rhodophyta) and the green seaweeds (phylum Chlorophyta). Each grouping varies considerably in their morphology, physiology and biochemistry. The chemical composition of seaweeds depending greatly on factors like species, time of harvesting, light availability and water temperature. Seaweeds are a common sight along the coastal regions of the world and have enjoyed a long tradition of diverse use by mankind, having been used for centuries in food preparation and in traditional medicine. Some seaweeds are rich in protein, while other species produce secondary metabolites with various biological activities. [1]. An immense variety of activities are attributed to seaweed components and are well documented in the literature. Among the most common uses of seaweeds today are their use in the drugs, cosmetics and the food industries [2]. Ireland is an island off the west coast of Europe, with a temperate climate and a very significant coastline (7500 km), allowing access to a large source of seaweed [3]. In the literature review presented in this thesis, the potential of using seaweed-derived components to treat a variety of serious non-communicable diseases (NCDs) such as cancer, cardiovascular disease and diabetes was discussed. NCDs are becoming much more prevalent as society become increasingly sedentary and loving longer and some being less health conscience. Whole generations of people are exercising less, eating more and consuming alcohol and tobacco to a dangerous extent. These risk factors are the driving force behind the development of new cases of NCDs, which are becoming an enormous burden on global health services. Edible seaweeds are natural superfoods, containing copious amounts of complex carbohydrates, vitamins, minerals and oils. They produce biologically active secondary metabolites that exhibit a plethora of activities which can help alleviate and treat NCDs. Recently, NCDs have replaced infectious diseases as the number one cause of human mortality. Current medical treatments for NCDs rely mainly on drugs that have been obtained from the terrestrial regions of the world, with the oceans and seas remaining largely an untapped reservoir for exploration. Seaweed bioactive compounds have demonstrated such activities as anti-cancer, anti-oxidant, anti-inflammatory, and anti-diabetic [4]. It is only in relatively recent times that the scientific community has developed the capabilities to better understand the health benefits of seaweeds that our ancestors knew of anecdotally. Indeed, the literature presented here clearly demonstrates the plethora of novel bioactives that seaweeds offer. While

much of the research heralds the therapeutic effects from *in vitro* studies, the way has been laid to assess their efficacy *in vivo* through appropriate animal and human clinical trials.

The main organic components of seaweed are polysaccharides, proteins and lipids [5]. Most of the polysaccharide content of seaweeds contain complex bonds which cannot be broken down by human digestive enzymes [6, 7]. In brown seaweeds, the soluble dietary fibre polysaccharides are alginates, fucans and laminarins, while the insoluble fibres are essentially made of cellulose. In red seaweeds, the major polysaccharides include carrageenan, xylan and agarans [8], while in green seaweeds among the major polysaccharides is ulvan [9]. Bifidobacteria are a major grouping of beneficial bacteria that colonise the intestinal mucosa of healthy individuals. They exhibit several health-promoting effects such as antimicrobial activity against enteric pathogens, immunomodulation and reduced incidences of gut disorders. Bifidobacteria can metabolise many of the complex dietary carbohydrates that escape hydrolysis by mammalian digestive enzymes in the gut and reach the colon intact. Many non-digestible carbohydrates in the gut act as prebiotics and enhance the growth of the bifidobacteria [10]. As such, bifidobacteria are often targeted for prebiotic intervention [11]. A prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit [12]. As a first step in the study of the prebiotic properties of Irish seaweeds (Chapter 2) it was of great interest to see if the carbohydrates found in seaweeds could be successfully utilised by different strains of bifidobacteria, and, therefore, demonstrate a bifidogenic effect. Fifteen Irish seaweeds (ten brown seaweeds, three red seaweeds and two green seaweeds) were accessed for bifidogenic activity using an anaerobic *ex vivo* approach. The media used in study was a specially designed minimal media for bifidobacteria, containing all the nutrients required for growth except a major carbon source. By supplementing the media with each seaweed extract, any observed growth would be as a direct result of *Bifidobacterium* utilising seaweed carbohydrates contained within the extract. At an extract concentration of 2.5 mg/ml, significant stimulation of bifidobacterial growth was observed for several of the extracts, most notably the brown seaweeds *Fucus serratus* and *Pelvetia canaliculata*. The results of the initial screen showed that *F. serratus* was the most promising candidate for further bifidogenic investigation as it had the greatest overall effect on *Bifidobacterium*. A dose-response study was carried out, and it was found that a *F serratus* concentration of 1.25 mg/ml demonstrated the greatest bifidogenic stimulatory effect, and significantly outperformed the positive control (FOS) in some instances at the same concentration. Interestingly, an inhibitory effect was

observed with the *F. serratus,* extract at a concentration of 2.5 mg/ml. It was found that several of the brown seaweeds, including *F. serratus,* contained large concentration of phenolic compounds. Phenolic compounds such as phlorotannins are commonly produced in brown seaweeds and their presence at a high concentration is the likely explanation for the inhibitory effect observed. A limitation of this study was the nature of the cold-water extracts. Solid-liquid extractions, using cold water as the solvent, is a cheap and quick way of obtaining biologically active substances from raw materials such as seaweed. However, the method lacks appropriate post-extraction processing, which allows for the presence of unwanted compounds such as phenols and monosaccharides to remain in the extractions. For reliable prebiotic investigations, an extraction method solely designed for the isolation of polysaccharides and the removal of unwanted materials is required. Also, the choice of solvent (in this case cold-water) must be carefully considered. For example, sulphated polysaccharides from seaweeds are generally extracted using hot water or dilute acid/alkali [13]. The conclusion of this study was that different *Bifidobacterium* strain can use cold-water seaweed extracts as their sole carbon source and that the bifidogenic effect is greatest amongst brown seaweed species. The brown seaweed *F. serratus* proved to be the best candidate for further prebiotic investigation, which would require the creation of a new type of seaweed extract. A refined extraction process, with appropriate post extraction processing to remove monosaccharides, phenolic compounds and other biologically active compounds found in seaweeds is recommended.

To further evaluate the prebiotic potential of *F. serratus* a polysaccharide-rich extract (Fse extract) was produced (Chapter 3) using an appropriately designed extraction process. Dilute hot-acid was used as the extraction solvent to aid the extraction of *F. serratus* structural polysaccharides with the extraction being repeated twice to maximise recovery. Following the primary extraction, several processing steps were used to address the issues encountered while using the cold-water extracts previously. Ethanol precipitation was used to isolate and separate the algal sugars from the remaining bulk. Monosaccharides and other small sugars were then removed from the extract by size exclusion dialysis, using 1 kDa cut-off dialysis tubing. Finally, a simulated gastric digestion was carried out to simulate passage from the mouth to the colon. The effects of prebiotics can be evaluated on the basis of the growth of probiotic bacteria such as bifidobacteria and lactobacilli, a decrease in intestinal pathogens, and a change in the production of health-related bacterial metabolites such as short-chain fatty acids (SCFAs), with the gold standard of prebiotic comparison studies being fructooligosaccharides (FOS) [14, 15]. It has been shown that many strains of *Bifidobacterium* are capable of fermenting FOS, whereas

only a minority can grow on inulin. Interestingly, both FOS and inulin have been proved to be bifidogenic, likely as a result of cross-feeding in faecal cultures [11]. The prebiotic potential of the Fse extract was assessed by an *ex vivo* anaerobic batch fermentation, using freshly voided faecal matter from healthy volunteers, allowing for observations to be made about changes in the main bacterial groups present within. Because of the inhibitory effect observed with cold-water extract of *F. serratus* in Chapter 2, a reduced concentration of 1% (w/v) was used. This concentration is in agreement with a study carried out by Ramnani et al. [16], which also used a batch fermentation approach to access prebiotic potential. Samples were taken for SCFA analysis, DNA sequencing, enumeration of *Bifidobacterium* and *Lactobacillus*, and the production of hydrogen sulphide (T = 0 h, 5 h, 10 h, 24 h, 36 h, and 48 h). The results from the fermentation of *F. serratus* polysaccharides were mixed. While significant increases in the production of SCFAs was observed, particularly the production of propionate and acetate, the fermentation of *F. serratus* polysaccharides had no measurable impact on bifidobacteria or lactobacilli. SCFAs are the major end-product of bacterial fermentation of undigested carbohydrates in the colon, and consist mainly of propionate, acetate and butyrate [17]. SCFAs mediate their influence by activating cells via cell surface G-protein-coupled receptors such as FFAR 2 (GPR44) and FFAR 3 (GPR41), which are differentially expressed by epithelial cells, adipocytes and phagocytes [18]. The production of SCFAs indicate that the polysaccharides contained in the Fse extract survived the simulated gastric digest intact and were fermentable by the representative microbiota. DNA sequencing of the 16s rRNA gene variable V4 region indicated a marked increase in the relative abundance of the propionate-producing superfamily *Veillonellaceae,* the genus *Parabacteroides* that is known to produce both propionate and acetate and the family *Erysipelotrichaceae* which is peripherally related to the butyrate-producing superfamily *Lachnospiraceae*. A significant shift ($p<0.05$) was observed in favour of the production of propionate. Non-significant increases in butyrate production were also observed. SCFAs have been shown to stimulate the release of the anorectic gut hormones peptide YY (PYY) and glucagon like peptide-1 (GLP-1) from rodent enteroendocrine L cells. These hormones are involved in the short-term signalling of satiation and satiety to the appetite centres of the brain. It is believed that SCFAs stimulate the release of GLP-1 in rodents by stimulating FFAR2. Propionate has the highest affinity for this receptor amongst the SCFAs [19]. Fermentation of the FOS control, a known prebiotic, resulted in a significant increase ($p<0.05$) of all three biologically significant SCFAs, acetate, butyrate and propionate. However, no significant effect was observed on bifidobacteria and lactobacilli populations

from DNA sequencing results or direct plate count enumeration. A notable non-significant increase in bifidobacteria numbers was observed at 10 h on MRS_{cys} plates. Hydrogen sulphide is a toxic substance produced by several genera of colonic bacteria [20] by the action of sulphate-reducing bacteria on inorganic sulphur or through the fermentation of sulphur containing proteins and amino acids such as methionine, cysteine, cysteine and taurine [21]. A reduction in the production of hydrogen sulphide is indicative of prebiotic action as carbohydrate fermentation would be seen to predominate over protein fermentation. No hydrogen sulphide was detectable in fermentate samples collected, precluding an assessment on the effect on hydrogen sulphide production. A major limitation of the study was that the amount of Fse extract, obtained from the extraction process, only allowed for the batch fermentation to be carried out three times. This reduced the statistical power of the data but, nonetheless, a clear insight into the prebiotic potential of *F. serratus* polysaccharides was obtained. A re-examination the prebiotic potential of *F. serratus* using a higher concentration of extract and an increased number of technical and biological repeats could yield a more robust result, leading to an *in vitro* animal trial focusing on the effect on the model microbiota and the release of satiety-related hormones (PYY, GLP-1). Further, the effect of *F. serratus* polysaccharides on bifidobacteria would be better evaluated using a qPCR approach using bifidobacteria specific primers targeting housekeeping genes such as *groEL, recA,* and *dnak* [22] and *rpoB.* This would also allow for species and strain diversity to be analysed [23]. The conclusion of this study was that polysaccharides from *F. serratus* do not exhibit prebiotic activity in agreement with the current definition. However, a shift in fermentation patterns towards the production of propionate can have a positive effect in relation to improving satiety and protecting against obesity. Further research into the prebiotic potential of *F. serratus* polysaccharides is recommended culminating in animal trials.

The biological function of polysaccharides is greatly dependent on their molecular weight. High molecular weight polysaccharides have more complex structures and conformations than lower molecular weight polysaccharides, and have a higher viscosity and poorer water-solubility. This makes it difficult for them to enter the interior of the cell or attach to receptors [24]. To date, there have been a limit number of studies regarding the degradation of seaweeds by the gut microbiota. Seaweed polysaccharides tend to be high molecular weight, resulting in a quick passage through the gut, reducing the time that they are available as fermentable substrate to the microbiota. Evidence is now emerging that low molecular weight polysaccharides and oligosaccharides can act as a source of fibre and a putative source of

prebiotics [16]. In nature, polysaccharides are often transformed into lower molecular weight oligosaccharides by a variety depolymerisation processes. To access the effect of depolymerisation on prebiotic potential two similarly processed polysaccharide rich extracts were created from the Irish seaweed *Laminaria digitata* (Chapter 4). One extract (the crude extract) was processed in an identical manner as the previous *F. serratus* extract, while the other (the depolymerised extract) was depolymerised using hydrogen peroxide. The effect of depolymerisation would be to lower the molecular weight of the parent *L. digitata* polysaccharide. Again, an anaerobic batch faecal fermentation was used to access prebiotic potential, with samples being taken for SCFA analysis, DNA sequencing, *Bifidobacterium* and *Lactobacillus* enumeration and hydrogen sulphide formation (T = 0, 5, 10, 24, 36, and 48 hours). The fermentation of both extracts resulted in significant increases in the production of all three biologically important SCFAs, butyrate, propionate and acetate. This indicates that *L. digitata* polysaccharides can survive a simulated gastric digestion, satisfying an important aspect of the prebiotic definition. Depolymerisation of *L. digitata* resulted in significant difference in SCFA production. The depolymerised extract exhibited significantly increased propionate production and decreased production of butyrate in comparison with the non-depolymerised extract. The most obvious reason for the stimulation of butyrate production *ex vivo* is the direct selection of members of the representative microbiota capable of using the substrate [25], in this case *L. digitata* derived polysaccharides. Genes for butyrate production are widely spread among anaerobic Gram-positive members of the microbiota including several families belonging to the phylum Firmicutes, *Roseburia* spp. family *Lachnospiraceae*), *Eubacterium rectale* (family *Lachnospiraceae*) and *Faecalibacterium prausnitzii*-related bacteria (family *Clostridaceae*) [26]. An increase in butyrate production is of great interest to researchers and health professionals. Butyrate is the preferred energy source for epithelial cells of the colon and also has been implicated in the prevention of colitis and colorectal cancer [27]. As with the *F. serratus* fermentation, no noticeable impact was recorded for either extract on bifidobacteria or lactobacilli. The selective stimulation of beneficial members of the gut microbiota underlines the prebiotic concept as currently defined and precludes the labelling of *L. digitata* polysaccharides as prebiotic at this time.

Previous fermentation studies of seaweed prebiotic potential mainly focused on the brown seaweeds, (*F. serratus* and *L. digitata*). It was of great interest to examine the potential of another grouping of seaweeds, the red seaweeds. The polysaccharide content of red seaweeds is fundamentally different from both the brown and green seaweeds consisting

mainly of carrageenan, xylans and agarose. *Chondrus crispus* was chosen as the model red seaweed as it is both an economical and historically important seaweed in Ireland (Chapter 5). The *C. crispus* extract (Cc extract) was prepared using a dilute hot-acid extraction and appropriate post-extraction processing. Owing to the gelling nature of the *C. crispus* polysaccharides, depolymerisation, using hydrogen peroxide, was required. The compositional analysis of the Cc extract, following treatment with TFA, revealed the presence of large quantities of galactose. The hydrolysis of carrageenan and agar is known to liberate galactose and 3, 6-anhydro-galactose [28]. This indicates the presence of large quantities of carrageenan in the extract as carrageenan can comprise 50-65% of the dry weight of *C. crispus* [29]. SCFA analyse of the Cc extract fermentation revealed that the extract was poorly fermented by the representative microbiota. Overall, only moderate increases in SCFA production was observed, albeit still significantly increased over the cellulose negative control. As with the fermentation of *F. serratus* and *L. digitata*, there was no effect on recovered *Bifidobacterium* and *Lactobacillus* populations. The poor prebiotic performance of the Cc extract could be because of the depolymerisation process itself degrading the polysaccharides beyond the point of been utilised by the representative microbiota. It would have been of interest to compare prebiotic activity between the depolymerised *C. crispus* extract and a *C. crispus* extract not having been subjected to a depolymerisation process. It was concluded that red seaweed polysaccharides are not suitable as candidates for further prebiotic investigation.

Foodborne illnesses are a major drain on public health services throughout the world. Such illnesses are common and often preventable, and affect approximately 30% of individuals in industrialised countries each year [30] [31]. In Chapter 6, the antimicrobial activity of ten Irish brown seaweed species were investigated. Extracts from brown seaweeds were selected because these are reported to contain more biologically active substance than red seaweeds (which are rich in protein) and green seaweeds. The pathogens *L. monocytogenes, S. aureus, E. coli* and *S. enterica* serovar Typhimurium were chosen because they are among the most prevalent foodborne organisms, which not only affect the quality of food but also cause severe illness if contaminated food is ingested [2]. The initial antimicrobial screen was carried out at a working concentration of 1 mg/ml and demonstrated that several of the ethanol extracts harboured potent activity against the food pathogen *Listeria monocytogenes*. The extracts that demonstrated the strongest effect were by *F. vesiculosus, F. spiralis, F. serratus, A. nodosum* and *P. canaliculata*. The greatest anti-listerial effect was observed with *F. vesiculosus.* Plate counts for surviving bacteria at 24 h resulted in the recovery of no viable colony forming unit

at any dilutions. *Fucus vesiculosus* was subsequently chosen for further investigation. Several different sub-fractions of *F. vesiculosus* were prepared. It was found that anti-listerial activity was concentrated in the 0 – 3.5 kDa and 3.5 – 100 kDa. Both fractions and the crude ethanol extract of *F. vesiculosus* inhibited the growth of all *L. monocytogenes* strains tested. Activity with the 3.5 – 100 kDa extract was observed against *Listeria* at a concentration of 10 µg/ml. Anti-listerial activity was positively correlated with phenolic content, with all extracts and fractions containing highest concentrations of phenolic compounds also demonstrating highest levels of antimicrobial activity. Phenolic compounds are a group of hundreds of different molecules that have a benzene ring in their structure substituted by at least one hydroxyl group [32]. As high-molecular weight phlorotannins appear to be, at best only weakly antibacterial and antifungal, intermediate- and low-molecular weight fractions are presumably responsible for the observed levels of activity against *Listeria* [33]. The highest observed levels of phenolic compounds were observed for *F. vesiculosus, F. spiralis, F. serratus, A. nodosum* and *P. canaliculata,* all of which displayed potent anti-listeria activity. Apart from activity against *Listeria,* several of the other seaweed extracts demonstrated moderate activity against the Gram-positive bacteria *S. aureus,* against the Gram-negative bacteria *E. coli,* and *S. enterica* serovar Typhimurium at 24 h**.** The observed activity of phenol-rich seaweed rich extracts and fractions is not surprising as the presence of large quantities of phenolic compounds were inferred to have caused the inhibitory effect observed in Chapter 2 with the cold-water extraction from *F. serratus.* Despite efforts to eliminate *L. monocytogenes* from ready-to-eat (RTE) foods, contamination still occurs. It has been estimated that as much as 5% of RTE foods, such as sliced luncheon meats and prepared deli-style salads, contain *L. monocytogenes* [34]. In many respects, *L. monocytogenes* differs from most known foodborne pathogens in that it is ubiquitous, resistance to diverse environmental conditions, and is microaerophilic and psychrophilic [35]. The ability of *Listeria* to persist in RTE meat processing plants is in part because of their ability to form biofilms that aids in resistance to sanitation efforts and allows the organism to be a persistent contaminant [36]. The conclusion of the antimicrobial screening of ethanol extract from brown seaweeds is that they can be potentially used to great effect as biopreservatives in the food industry, especially phenolic-rich *F. vesiculosus* derived agents against *L. monocytogenes*.

In conclusion, the potential of seaweeds to positively impact on the health of individuals is enormous. Through the modification of SCFA production, it can be expected that seaweed polysaccharides can improve overall gut health by increasing the production of butyrate,

propionate and acetate either by direct consumption or by their incorporation into other food stuffs. Butyrate is a major source of energy for colonocytes and well regarded as a protectant against colorectal cancer and other gut disorders, while propionate is positively associated with satiety and glucose regulations. To qualify as a prebiotic, several criteria must be met by the substrate being investigated, namely the ability to resist gastric acidity and hydrolysis in the upper regions of the gastrointestinal tract and to be fermentable upon reaching the colon, leading to the selective growth and/or stimulation of a limited number of beneficial microbes. Observed increases in the production of SCFAs indicate that a multitude of seaweed polysaccharides can survive gastric digestion and reach the colon intact and be fermented on by colonic bacteria. None of the seaweed fermentations carried out resulted in a positive effect on *Bifidobacterium* and *Lactobacillus*. Seeing that positive impacts on SCFA production were observed for both the *F. serratus* and *L. digitata* fermentations, this could be a failure of the methods used to quantify the effect on beneficial members of the microbiota. FOS is a well-known and established prebiotic and is often cited in literature as a control in prebiotic studies. For all FOS fermentations (*F. serratus* study, *L. digitata* study, and *C. crispus* study) as expected, significant increases in acetate, butyrate and propionate were recorded, however, no clear stimulation of *Bifidobacterium* or *Lactobacillus* was evident. Alternatives to traditional culturing methods would add greatly to prebiotic investigations. Methods such as the plating of serial dilutions on the selective media is both laborious, time consuming and prone to large degrees of error. Replacement with molecular technologies, such as qPCR, and through utilising primers designer to target specific members of the microbiota, would allow for the collection of unequivocal data about the effect of putative prebiotics. Time constraints precluded a more extensive use of molecular techniques in this thesis. Seaweeds are clearly an excellent source of dietary fibre, the benefits of which are well documented in literature. Based on the results present here further investigation of brown seaweed as a source of prebiotics is recommended. Seaweeds components have clearly been established to have a wide range of biologically active components. They contain a multitude of non-digestible polysaccharides that can reach the colon intact and be fermented by colonic bacteria while also contain compounds such as polyphenols that are proposed to possess potent antimicrobial activity. This underlines the importance of a properly designed extraction methodology to more efficiently isolate these compounds with opposing activities. The development of new biopreservative agents utilising phenolic compounds isolated from seaweeds such as *F. vesiculosus*, and other closely related species, could have a profound impact on the shelf life and overall quality of

RTE foods, especially as a protectant against *Listeria* contamination. Once the safety of ethanol and phenolic extractions from seaweeds is proven, using mammalian cell culture models followed by animal and human trials, incorporation of seaweed extracts into food matrices for human consumption has strong potential as functional food ingredients to promote health of consumers.

7.2 References.

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